Controlled release of Brain Derived Neurotrophic Factor to promote neuron survival following chronic electrode implantation

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Aswin K. Sundarakrishnan

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Dedications

I would like to dedicate my work to my parents, Mani Sundarakrishnan and Radhika Sundarakrishnan who have really been supportive of all my ventures. They have really been there for me at both times of stress and joy. I would also like to dedicate this work to my loving brother Aaryaman Sundarakrishnan and sister Adithi Sundarakrishnan.

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List of Abbreviations

PNS – Peripheral Nervous System

CNS – Central Nervous System

AP – Action Potential

GFAP – Glial fibrillary acidic protein

TNF-α –Tumor Necrosis Factor-[alpha]

INF-β –Interferon-[beta]

IL-1 –Interleukin-1

DRG –Dorsal Root Ganglion

PLGA – poly (lactic-co-glycolic acid)

FDA –Food and Drug Administration

LN –Laminin

PEI –Polyethyleneimine

LbL –Layer-by-layer

BDNF –Brain Derived Neurotrophic Factor

NGF –Nerve Growth Factor

NT-2/3/4 – Neurotrophin-2/3/4

GDL – (D-glucono-delta lactone)

PEO – Polyethylene oxide

PPO - Polypropylene oxide

Abstract

Controlled release of Brain Derived Neurotrophic Factor to promote neuron survival following chronic electrode implantation

Aswin K. Sundarakrishnan

Advisors: Karen Moxon, PhD, Margaret A. Wheatley, PhD

Chronic electrode implantation in the brain leads to the formation of a glial scar surrounding the implantation site. The glial scar, when formed, severely inhibits the long term in-vivo functionality of neural electrodes. Studies in the past have tried coating the neural electrodes using polymers with or without bioactive agents. However none report any success in alleviating the glial scar or show improvement in electrode recording capability. Previous studies have suggested the use of neurotrophin delivery from electrode coatings to promote neuron survival and improve electrode biocompatibility. In the current work, the feasibility of delivering Brain Derived Neurotrophic Factor (BDNF) from alginate thin film coatings on silicon electrodes was evaluated.

Electrode coatings were manufactured via spinner coating using 0.45 μm filtered 2% (w/v) or 4% (w/v) alginate solutions on silicon wafer substrates. To crosslink the alginate two different methods of crosslinking were evaluated, the fast $CaCl_2$ crosslinking and the slow $CaCO_3$-GDL crosslinking method. However $CaCl_2$ crosslinking using an aerosolized spray produced consistent alginate coatings compared to the $CaCO_3$-GDL crosslinking method and was subsequently used for all experiments. Thickness measurements using the profilometer showed that $CaCl_2$ crosslinked alginate

coatings in the micron level can be produced by spin coating 0.45 μm filtered, 4% alginate solutions at 1000 rpm for 30-60 seconds. When multiple layers of alginate were applied on top of one another, coatings of thicknesses ranging from 5 μm to 80 μm were produced via spinner coating. To simulate BDNF release, all alginate films were loaded with either model proteins (lysozyme or chymotrypsinogen) or poloxamer (p-188), a polymer that has been shown to have membrane-sealing properties. In-vitro release studies from all loaded films were conducted in PBS (pH 7.4) at 37°C until all protein or poloxamer was released.

Results from the release studies showed rapid release profiles for both model proteins and p-188. Complete release of proteins and p-188 took place within the first three hours. This is well short of the anticipated 3-4 day release period required for BDNF. In order to prolong release for BDNF, the effect of addition of a poly-L-ornithine (PLO) coating on the alginate layer was evaluated. PLO coatings at three different concentrations, 0.1(w/v) %, 0.5% and 1% were applied on the surface of alginate films. Subsequent release studies showed very similar release profiles between the PLO-coated and the uncoated films, and were probably due to the detachment of the film from the silicon surface early in the release.

The results of this study clearly show the feasibility of manufacturing neurotrophin releasing alginate coatings for silicon electrodes. The versatile coating method can be used to deliver a number of different compounds including proteins like neurotrophins and polymers like p-188. However to achieve controlled release over

longer periods of 3-4 days, alternate coating methods will need to be investigated.

1 INTRODUCTION

1.1 Neural Implants

Every year patients in the millions lose neural function due to traumatic injury (i.e., brain injury or spinal cord injury), congenital conditions, degenerative diseases (e.g., retinis pigmentosa or age related macular degeneration) or other neural disorders (Winter O. J., 2007). While damage to the Peripheral Nervous System (PNS) is often repaired naturally, damage to the Central Nervous System (CNS) cannot be repaired leading to permanent disability of the individual. However studies have shown that neural pathways upstream of injury remain active (Winter O. J., 2007). Neural implants could therefore be used to tap into these pathways to regain functionality (Winter O. J., 2007).

A good example of a neural implant that is in used today is the prosthetic cochlear implant that is used for the treatment of deafness (Bell et al., 1998). In a normal ear of an individual, the hair cells receive a wave of vibrations which they then convert into action potentials in the adjacent auditory neurons (Bell et al., 1998). These action potentials are then taken to the CNS to be perceived as sound. In deaf people, cochlear implants bypass the damaged hair cells stimulating the auditory neurons directly, therefore completing the neural circuit to the CNS (Bell et al., 1998).

However neural implants do have some disadvantages. One of the major problems with neural implants is their failure to provide long term in-vivo

recordings. Two major reasons have been noted for the failure of such implants (i) the mechanical failure of the implant itself: failure of the cable from the electrode to the signal conditioning device or loss of polymeric insulation of the microelectrode due to the corrosive extracellular environment and (ii) the formation of the glial scar surrounding the region of implantation rendering the implant useless because of its inability to record action potentials from neurons (Moxon K. A., 2007). While the former has been resolved the latter continues to be a major drawback for the long term in-vivo functionality of neural implants.

1.2 Overall Objective

The overall goal of this project was to improve the long term in-vivo functionality of silicon electrodes. To achieve this end goal we focused on improving the biocompatibility of the silicon electrodes by providing controlled release of Brain Derived Neurotrophic Factor (BDNF) from alginate electrode coatings. Currently there are very few studies that have successfully shown neurotrophin delivery from neural electrodes. Studies which have successfully delivered neurotrophins in the past have failed to optimize delivery parameters including, but not limited to the method of delivery, the amount of protein to be delivered and length of delivery. The specific aims of this particular project are as follows…

- To determine the ability of spinner coating to produce alginate electrode coatings. The aim is to establish the right combination of material parameters

and spinner coating parameters required for manufacturing alginate electrode coatings of the required thickness.

- To evaluate the feasibility of loading and releasing model proteins, lysozyme and chymotrypsinogen from alginate coatings. The aim is to figure out the appropriate method of protein loading and the feasibility of loading physiologically relevant quantities of protein in the alginate coatings.
- To test if the coating is versatile and is able to deliver other neuroprotective substances like poloxamer (p-188).
- To test the ability of poly-L-ornithine (PLO) coatings on alginate to provide controlled release of the model proteins and poloxamer (p-188).

2 DESIGN ASPECTS

2.1 Design Criteria

2.1.1 Electrode coating material

- The aim of the current study is to show in-vitro BDNF release from alginate coated silicon wafer substitutes. It is expected that the in-vitro studies from this project will pave way for the development of electrode coatings eluting protein in-vivo. Therefore the coating material used for the project would have to be extremely biocompatible. Also, the chosen material would have to biodegrade naturally without any cytotoxicity when implanted in-vivo.

- The material chosen for manufacturing the coatings would have to be easily available.
- Previous studies have shown that material-protein interactions may exist (Wells and Sheardown, 2007). Some interactions are harmless while some others have been shown to lead to changes in the protein conformation. For example Transforming Growth Factor – β1 (TGF-β1) encapsulated in alginate beads have been shown to become inactive due to alginate-protein interactions (Gombotz and Wee, 1998). In comparison alginate-protein interactions were found to enhance bioactivity of Vascular Endothelial Growth Factor (VEGF) following encapsulation (Peters, 1998). Hence the chosen coating material should not render the protein (BDNF) inactive.
- Cost will be a factor if more than one material is identified with proven therapeutic use. In such a situation the least expensive material will be chosen for manufacturing coatings.

2.1.2 Coating method

- Reproducibility of the coatings produced using the method will be important for future commercialization and use. Any variability in coating thickness must be at a minimum, if any.
- The method chosen for manufacturing electrode coatings should be cost effective for it to be used on a frequent basis.

Table 2.1: Comparison of different methods for manufacturing electrode coatings

Coating method	Thickness of films produced	Advantages	Disadvantages	References
Solvent casting	microns - few millimeters	(i) Films of uniform thickness can be produced	(i) Mold according to the dimensions of the electrode (micron scale) may be required	(Lu P., 2001; Siemann, 2005)
Electrospinning	nanometers - millimeters	(i) Consistent micron level films can be produced	(i) High voltage application may cause denaturation of protein dissolved in polymer solution (ii) Electrospinning of alginate alone is not possible, need an additive	(Schiffman and Schauer, 2008)
Spinner coating	nanometers - microns	(i) Extremely simple to use (ii) Thin films of the required thickness can be produced using this method	(i) If protein is mixed with polymer and spinner coated, wastage of protein may occur	(Cathell and Schauer, 2007)
Layer-by-Layer (LbL)	angstroms - nanometers	(i) Extremely simple process	(i) Films don't break down in 1xPBS. Need harsh ionic environments to fall apart (ii) micron level coatings may not be possible	(He W., 2005; Ren K., 2005; He W., 2006)

2.2 Design Constraints

2.2.1 Electrode coating

- The electrode coatings that are manufactured should be as thin as possible. They should not exceed the dimensions of the electrode itself. Therefore the coatings should not exceed 50μm in thickness. A thicker coating could potentially inflict more damage to the brain tissue.
- The electrode coatings manufactured using the chosen method should allow for loading of physiologically relevant quantities of protein. To calculate the amount of BDNF that would need to be loaded into the electrode coatings a literature search (Table 2.2) was performed, identifying studies that delivered BDNF to promote neuron survival. Information from these studies like volume of injury (mm^3), amount of BDNF delivered (μg/hr) and length of delivery (days) were used to calculate the amount of BDNF that would need to be delivered to promote neuron survival following silicon electrode implantation (Appendix-A).

Table 2.2: Literature search showing the amount of BDNF that would need to be delivered to promote neuron survival following silicon electrode implantation

Study details	References				
	(Giehl M. K., 1996b)	(Gillespie N. L., 2003)	(Tuszynski et al., 1996)	(Hammond, 1999)	(Vavrek et al., 2006)
Neuron Type	corticospinal neurons	auditory neurons	hypoglossal nerve	corticospinal neurons	corticospinal neurons
Volume of Injury Induced (mm^3)	52	N/A	N/A	52	N/A
When did delivery start?	Immediately after injury	5 days after ototoxin exposure	Immediately after injury	Immediately after injury	Immediately after injury
Amount of BDNF delivered (µg/hr)	0.5	0.016	0.05	0.5	0.5
Time of delivery (days)	7	28	14	14	14
End Result	promotes survival	promotes survival	promotes survival	promotes long term survival	promotes connections

3 IMPROVING THE BIOCOMPATIBILITY OF NEURAL IMPLANTS

3.1 Nerve Injury

When neural implants are inserted into the cortex they tear through nerve tissue, severing capillaries, extracellular matrix, glial and neuronal processes causing extensive damage at the site of electrode insertion (Polikov et al., 2005). The resulting mechanical damage triggers a foreign body response by activating platelets, clotting factors and macrophages. Immediately following the foreign body response the

body triggers a number of repair mechanisms to allow for fast recovery. However depending on the site of injury the repair may or may not occur.

3.1.1 Peripheral Nervous System (PNS) Injury

In the PNS any damage to the neurons is often repaired. Figure 3.1 shows a neuron in the PNS undergoing injury. As shown by the figure the axon of the injured neuron degenerates immediately following injury. Any axonal or myelin debris formed in the process is cleared by phagocytotic cells such as macrophages and Schwann cells. Once the debris is cleared, regeneration of the axon starts at the proximal end and continues towards the distal end to the target tissue (e.g., muscle fibers) as seen in the Figure 3.1.

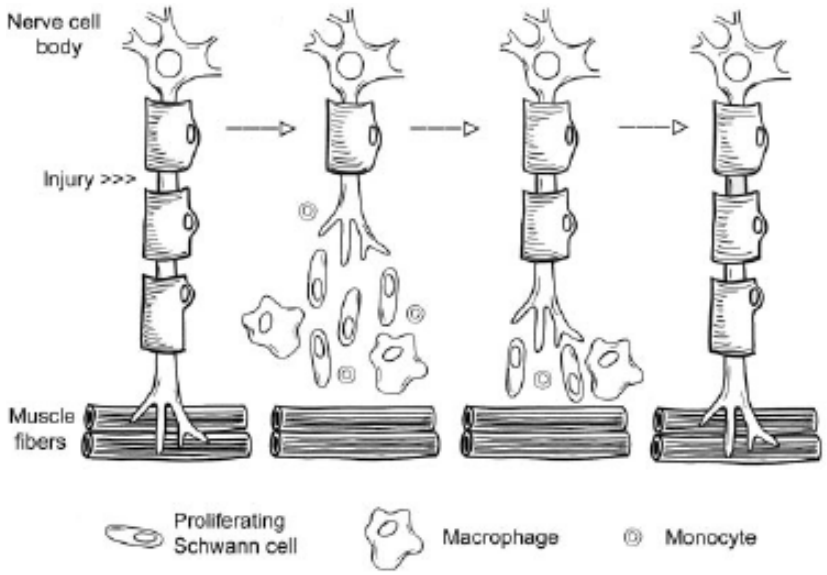


Figure 3.1: Regeneration of the axon following axotomy in the Peripheral Nervous System (figure reproduced from Schmidt and Leach, 2003)

3.1.2 Central Nervous System (CNS) Injury

Unlike the PNS, injury in the Central Nervous System (CNS) cannot be repaired. This is mainly due to the inability of the nerves to regenerate due to the presence of inhibitory glycoproteins present in the extra cellular environment of the CNS. Figure 3.2 shows a neuron in the CNS undergoing injury.

When a nerve is severed in the CNS, macrophage infiltration occurs just like in the PNS. However their recruitment is much slower compared to the PNS due to the blood-brain barrier, resulting in the delayed removal of the myelin debris at the site of injury (Schmidt and Leach, 2003). In addition to the above, up-regulation of cell adhesion molecules at the site of injury in the CNS is not comparable to the up-regulation seen in the PNS, therefore limiting macrophage recruitment further (Schmidt and Leach, 2003). Last but not the least of the problems, astrocytes found surrounding the region of injury in the CNS become reactive and form glial scars that inhibit any sort of regeneration from happening.

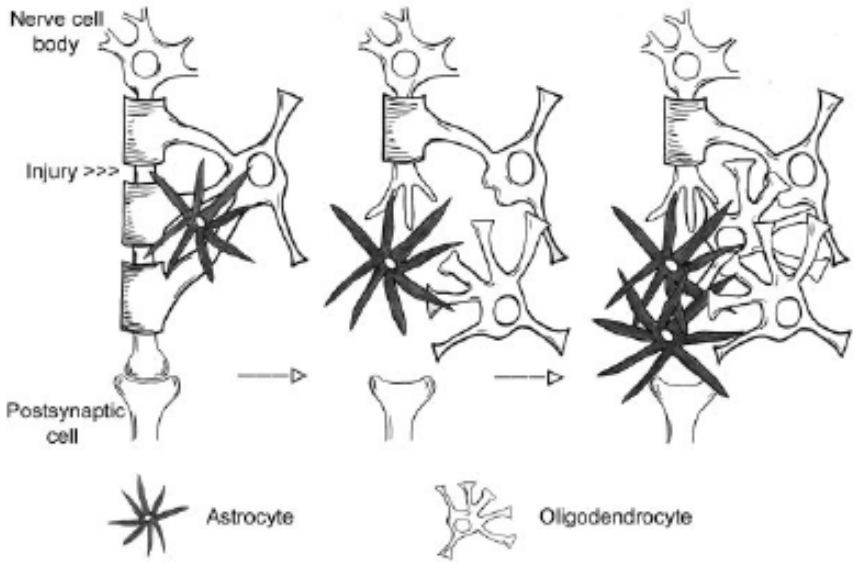


Figure 3.2: Regeneration of the axon following axotomy in the Central Nervous System (figure reproduced from Schmidt and Leach, 2003)

3.1.3 Glial Scar Formation

A number of studies have investigated the formation and progression of the glial scar. One study by Biran et al., studied the glial scar following chronic microelectrode implantation. In this study microelectrode implanted animals were compared to control animals which received only a stab wound using the same type of microelectrodes. At 2 and 4 weeks post-implantation immunostaining was performed to look for the presence of the different cell types including macrophages, astrocytes and neurons (Table 3.1).

Table 3.1: Summary of antibodies used for histology and immunostaining of brain tissue surrounding electrode implantation site (table reproduced from Biran et al., 2005)

Summary of antibodies

Antibody	Antigen	Cell type(s)
CD68 (ED1)	Lysosomal glycoprotein	Microglia, macrophages
CD11b/c (Mac-1)	CR3 complement receptor	Microglia, macrophages
GFAP	Glial fibrillary acidic protein	Astrocytes
Neurofilament-160	Medium neurofilament polypeptide	Neurons
NeuN	Neuronal nuclei	Neurons

Figure 3.3 shows the results obtained from the immunostaining performed at the electrode implantation site. Together, both Figure 3.3A and Figure 3.3B describe the spatial location of the different cell types surrounding the microelectrode. As seen in Figure 3.3, immediately surrounding the microelectrodes are the macrophages visualized by ED1+ and Mac1+ staining. The peak intensity of the ED1+ (microglia and macrophage) immunoreactivity was found to be within a 50μm radius (Figure 3.3) surrounding the microelectrode surface with no significant change between 2 and 4 weeks (Biran et al., 2005).

Compared to the macrophages (ED1+) the reactive astrocytes (GFAP+) were found starting at the 50μm radius extending over a distance greater than 500 μm surrounding the implantation site (Figure 3.3). The astrocytes formed a sheath-like encapsulating layer surrounding the macrophage rich zone, with neurons being largely excluded from these zones (Figure 3.3) (Biran et al., 2005). Also, similar to ED1, there was no significant change in the intensity of GFAP

staining surrounding the microelectrodes both at 2 and 4 weeks post-implantation.

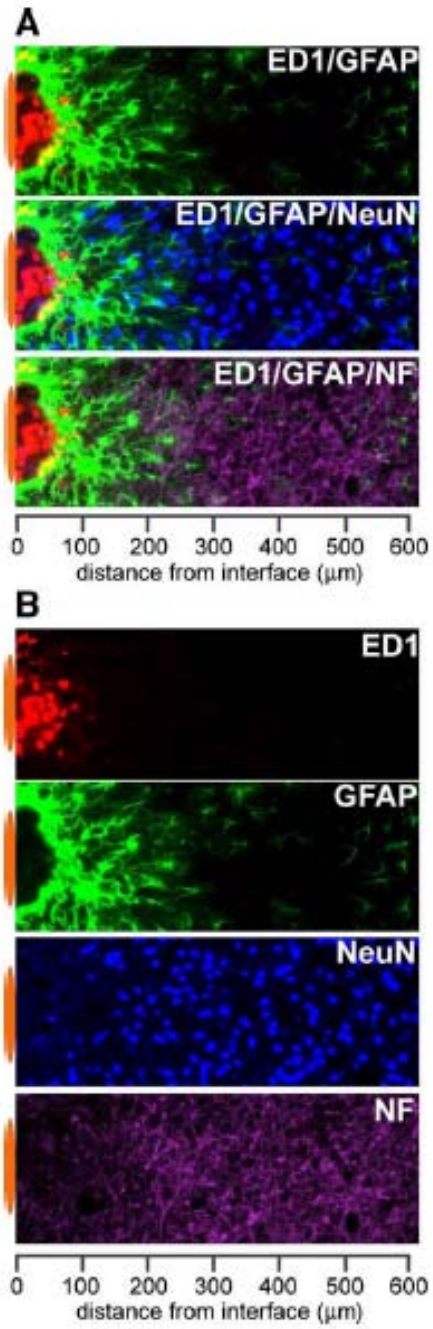


Figure 3.3: Immunostaining showing the spatial location of macrophages, astrocytes and neurons 4 weeks post-implantation (figure reproduced from Biran et al., 2005)

Staining for neuronal nuclei (NeuN) revealed an alarming ~40% reduction in the number of neuronal bodies within a 100μm radius (Figure 3.3) surrounding the microelectrode at 4 weeks post implantation (Biran et al., 2005). Apart from the reduction in the neuronal bodies a decrease in the neuron filament (NF) density and reactivity was also noticed up to 230μm surrounding the microelectrode surface. This reduction in neuronal density immediately surrounding the electrode is expected to be due to the death of neurons caused by the persistent inflammatory response consisting of activated microglia (Biran et al., 2005).

3.1.4 Neuronal Death

The inability of the neural electrodes to provide long term in-vivo recordings from neuronal processes is mainly due to the death of neurons immediately surrounding the electrodes. Therefore promoting neuron survival following electrode implantation will be important in ensuring electrode recording capacity.

Neuronal death following chronic electrode implantation occurs in a sequential manner due to a number of different events. The first series of neuronal deaths occurs during electrode insertion. When electrodes are inserted into the cortex they axotomize neuronal processes leading to death of neurons (Polikov et al., 2005). Although some neuronal damage occurs during electrode

insertion, most of the neuronal death is expected to be due to the inflammatory response produced by the recruitment of macrophages in the surrounding area. Biran et al., observed a strong inverse correlation between the NF (neurons) staining and ED1 (macrophage) staining, implying that (i) macrophage activation leads to neuronal loss or (ii) neuronal damage at the site of injury leads to high macrophage activation due to another mechanism or (iii) neurons are displaced from the electrode site by the inflammatory reaction (Biran et al., 2005).

According to Biran et al., the macrophage activation leading to a phenomenon called "frustrated phagocytosis" is one of the major reasons for neuronal loss surrounding the electrode. (Biran et al., 2005). The phenomenon is tagged as a positive feedback mechanism where the macrophages continuously produce cytokines due to their inability to clear the insoluble silicon electrode. The death of neurons is therefore due to the toxicity created by cytokines like TNF-α, IL-1β and prostaglandins (Biran et al., 2005).

A study conducted by Fitch et al. further provides support to this theory. Both studies are in agreement that the death of neurons is due to cytokine toxicity; however Fitch et al. suggests that some degree of mechanical damage also occurs to neurons due to astrocyte migration. Astrocytes are the primary support cells for neurons in the CNS (Schmidt and Leach, 2003) and migrate away from the macrophage rich zones. Therefore mechanical damage to the

neurons occurs as they were stretched, moved, pulled and torn between the migrating astrocytes (Fitch et al., 1999).

3.2 Biocompatible Neural Implants

Effective recording of action potentials from single neurons is possible only when the distance between the electrode and nearby neuronal cell body is of the order of cell dimensions, between 50 and 100 μm (Polikov et al., 2005). However with the glial scar forming at the very first day post-implantation neuronal death and movement occurs. Therefore there is a need for more biocompatible neural implants with long term in-vivo recording capacity.

A number of different approaches have been used in the past to solve this problem. One approach that has been studied is to improve electrode biocompatibility by coating it with biocompatible polymers. Poly (lactic-co-glycolic acid) (PLGA) coated electrodes have been manufactured in the hope of alleviating the glial scarring in the brain (DiPaolo C. B., 2003). PLGA, a highly biocompatible and FDA (Food and Drug Administration) approved polymer was used to coat ceramic silicon electrodes. To study the effect of the PLGA coatings on the glial scar formation, in-vivo studies were conducted in Long-Evans rats.

For the in-vivo study, coated silicon electrodes were implanted into the cortex of rats. Uncoated controls implanted into the cortex of the same animals were used for comparison. Immunohistochemistry (IHC) was then performed to look for the

presence of microglia, astrocytes and neurons surrounding the electrode implantation site.

The results from IHC showed no significant difference between the coated and uncoated electrodes (DiPaolo C. B., 2003). The number of glia surrounding coated electrodes and uncoated controls remained the same (DiPaolo C. B., 2003). Also, the healthy neurons continued to be isolated from the electrode due to the presence of the microglia and reactive astrocytes (DiPaolo C. B., 2003).

He et al., tried to alleviate the glial scar using a very similar, but different approach. In addition to the coating they also tried to immobilize laminin (LN), a bioactive protein. In comparison to DiPaolo et al., this study used a different method to coat the electrodes. Nano-coatings of polyethyleneimine (PEI)-laminin were manufactured using the layer-by-layer (LbL) approach. Using silicon wafers as the substrate, the authors created multilayers of oppositely charged PEI and laminin. Thickness measurements of the manufactured films showed that even coatings of up to 11 nm could be manufactured using the LbL technique (He W., 2005). To check the biocompatibility of the films produced, the authors performed two in-vitro assays, a neuron cell adhesion assay and a neurite outgrowth assay. The results from both assays showed that PEI-LN coatings were highly biocompatible (He W., 2005).

Having obtained positive results from the in-vitro studies, the authors carried out in-vivo studies in rats (He W., 2006). PEI-LN coated electrodes were implanted into the cortex of male Sprague-Dawley rats. As controls they implanted uncoated electrodes into the cortex of the same animals. Once again very little difference was

noticed between the LN-coated and uncoated probes 1 week after implantation. The LN coated probes did not reduce the accumulation of macrophages or reactive astrocytes in the first week (He W., 2006). Also no difference in neuronal proximity to the electrode recording site or neuronal density between bare and LN coated probes was observed (He W., 2006).

Failure of both the above studies clearly showed that coatings with or without protein/drug immobilization were not going to provide long term in-vivo functionality for electrodes. The coatings neither attracted neurons nor prevented the glial scar from forming. Recent studies have therefore shifted focus towards coatings that allow diffusion of pharmacological agent(s) or neurotrophins. Moxon et al., has suggested that delivery of neurotrophins could help ameliorate the short term impact of the microelectrode insertion and bind neurons to the microelectrode (Moxon K. A., 2007). One of the primary advantages of the drug delivery approach could be its ability to reach neurons beyond the glial scar region (He W., 2006).

3.3 Neurotrophin delivery

Neurotrophins are a family of proteins that regulate neuronal survival, axonal growth, synaptic plasticity and neurotransmission (Lu P., 2001). The neurotrophin family includes nerve growth Factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6, ciliary neurotrophic factor, basic fibroblast growth factor, glial derived neurotrophic factor, cholinergic development factor and other possible factors (Tuszynski et al., 1996).

Among these growth factors NGF, BDNF and NT-3 have been delivered to the brain to aid in neuronal survival and/or growth. BDNF has been shown to prevent injury-related motor neuronal degeneration (Tuszynski et al., 1996), adult basal forebrain cholinergic neuronal degradation (Tuszynski et al., 1996), and corticospinal neuronal degeneration following axotomy (Giehl M. K., 1996a). Table 2.2 shows a summary of some of the studies that report survival effects following BDNF delivery.

BDNF promotes neuron survival by binding to specific tyrosine kinase receptor – B (TrkB) in the target neurons. The receptor once activated has been shown to trigger one of two survival pathways, the PI3K pathway or the Erk pathway (Figure 3.4). While the former is straightforward and promotes survival directly the latter is dependent on infusion of calcium from calcium channels. Irrespective of the path taken, the final target of both pathways has been shown to be pro-apoptotic genes like Forkhead and Bad which facilitate neuronal survival (Sossin and Barker, 2007).

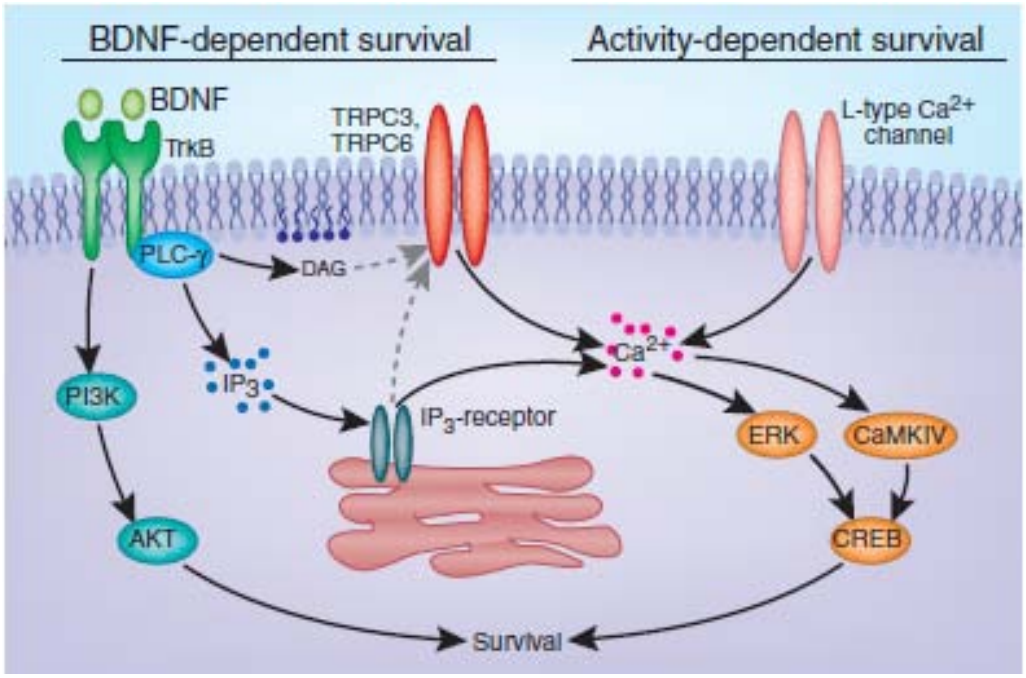


Figure 3.4: Pathways by which BDNF elicits its survival effects on neurons (figure reproduced from Sossin and Barket, 2007)

For this particular study we hypothesized that BDNF delivery could help promote long term in-vivo functionality of neural electrodes, by helping neuron survival in the short term. The only major drawback to BDNF delivery, which has been suggested in the past, is the half life of the protein in-vivo. The half-life of BDNF in the plasma was been found to be as low as 3 hours (Kishino et al., 2001). Therefore to overcome this problem controlled release of the protein from a polymer over a sustainable period of time has been suggested (Vögelin et al., 2006).

Vögelin et al., successfully showed the controlled release of BDNF using alginate microbeads ranging from 300-700 μm in diameter. In-vitro studies conducted by the authors showed that the alginate degraded slowly in phosphate

buffered saline (PBS) releasing BDNF for 16 days with a maximum of 25μg/ml per day (Vögelin et al., 2006). The authors also showed that the alginate helped maintain the bioactivity of the protein over time. The in-vivo studies showed that the BDNF, that was released, continued to support peripheral nerve regeneration at 4 and 10 weeks post administration (Vögelin et al., 2006). Therefore controlled release of BDNF from alginate matrices could promote neuron survival following electrode implantation in the CNS.

3.4 Poloxamer (p-188)

Another neuroprotective agent that has received lot of attention is poloxamer (p-188). Poloxamer is a water soluble, amphiphilic, triblock copolymer that has been approved by the FDA to be used a skin cleanser (Serbest et al., 2005). As shown in Figure 3.5 the polymer has two hydrophilic polyethylene oxide residues (PEO) on either side and a polypropylene oxide (PPO) residue in the center.

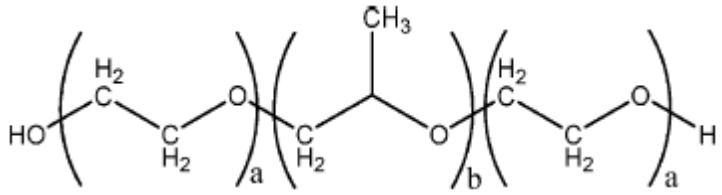


Figure 3.5: The chemical structure of poloxamer (p-188), a polymer with membrane sealing properties a=80, b=27 (figure reproduced from Mao et al., 2004)

Poloxamer when added to mechanically injured cells, has been found to seal plasma membranes, promoting cell viability and hence subsequent recovery (Serbest

et al., 2006). Serbest et al., treated mechanically injured PC12 cells, a subline of rat pheochromocytoma cell line with p-188. Results from the in-vitro study showed that p-188 promoted neuronal cell viability in a dose dependent manner. For the current study we hypothesize that neurotrophin delivery along with poloxamer could enhance neuron survival following electrode injury.

3.5 Alginate for drug delivery

The use of alginates as drug delivery vehicles is well documented. A number of studies have successfully shown controlled release of proteins from alginate matrices. Growth factors including Nerve Growth Factor (NGF), Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor – β (TGF-β) have been delivered in the past using alginate hydrogels (Gombotz and Wee, 1998).

Alginate, extracted from brown seaweed is a water-soluble, biocompatible, biodegradable, and inert biopolymer (Tobias et al., 2001). The linear polysaccharide is composed of repeated units of (1-4)-α-L-guluronic acid (G unit) and (1-4)-β-D-mannuronic acid (M unit) in varying proportions (Figure 3.6) (Tobias et al., 2001). Within the linear polysaccharide the M and G monomers are sequentially assembled in either repeating (MM or GG) or alternating blocks (MG). Figure 3.6 shows the structure of L-guluronic acid and D-mannuronic acid residues.

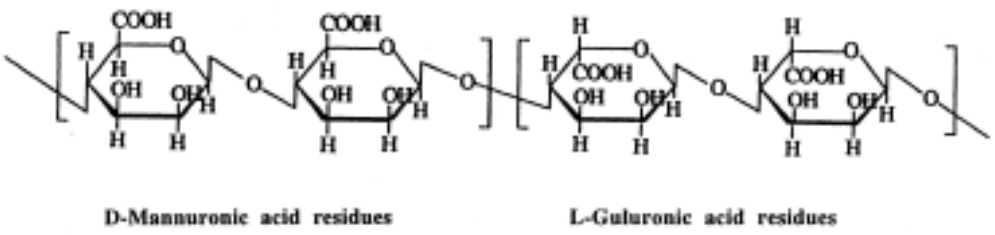


Figure 3.6: The chemical structure of L-guluronic acid and D-mannuronic acid residues of alginate (figure reproduced from Gombotz and Wee, 1998)

When sodium alginate is mixed with water at lower concentrations (< 2% (w/v) alginate in distilled water) it remains relatively liquid, but at higher concentrations (>2% (w/v) alginate in distilled water) the solution becomes extremely viscous and forms a physical gel. The viscosity of alginate solutions however is largely dependent on the molecular weight of the alginate used (Gombotz and Wee, 1998).

In comparison to alginate solutions, crosslinked alginate gels have been found to have superior mechanical properties including longer degradation times' in-vivo. Crosslinking of alginate is possible by exposing alginate solutions to divalent cations like Ca2+. The Ca2+ ions replace the sodium ions from the guluronic acids resulting in the formation of an egg box structure (Figure 3.7). Subsequently dimerization of alginate chains occurs resulting in the formation of a gel like network.

Crosslinking of alginate is reversible. When crosslinked alginate gels are placed in a saline environment (similar to in-vivo body conditions) the Ca2+ ions are removed leading to de-crosslinking and de-stabilization. Any protein encapsulated in the crosslinked gel is released during this time. However de-crosslinking and degradation is not the only method of protein release from alginate gels. Protein

release has also been shown to occur by diffusion, through the network pores of the crosslinked gels.

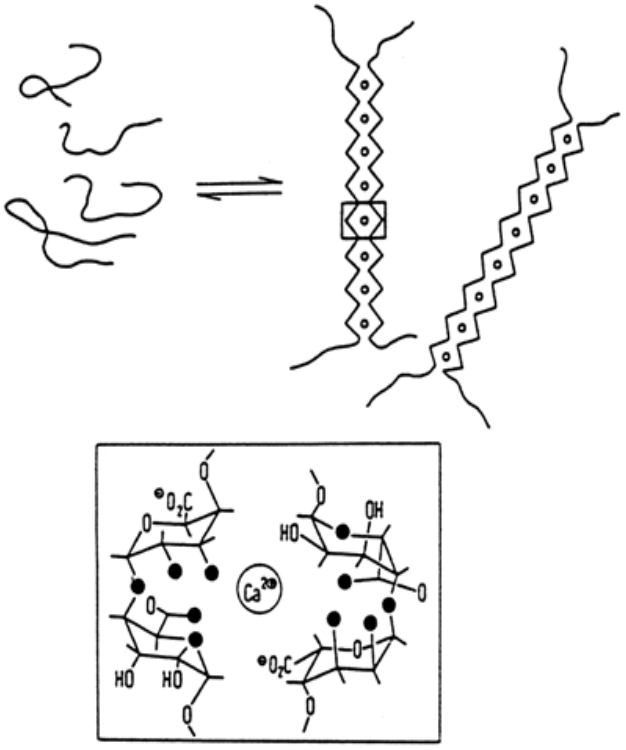


Figure 3.7: Eggbox structure formed during crosslinking of alginate following addition of divalent cations like Ca2+ (figure reproduced from Gombotz and Wee, 1998)

One of the great advantages of using alginates for drug delivery is the ability to modify protein release by altering their material properties. In the past, material properties of alginate like concentration, molecular weight, pH, temperature and

crosslinking method have been varied to optimize protein release. Apart from material properties, several studies have used polycation coatings on crosslinked alginate gels to alter network pore size and degradation rate. Tobias et al., used poly-L-ornithine (PLO) coatings on alginate beads to alter network pore size. In this study, genetically modified fibroblasts producing BDNF were encapsulated in alginate beads coated with PLO. The PLO coatings in this study were semipermeable. While allowing exchange of nutrients and waste products in and out of the alginate beads the coatings also provided immunoisolation. Due to the smaller network pore size they were able to selectively block any host immune cells from entering the beads, therefore providing isolation from the host's immune system.

4 MATERIALS AND METHODS

4.1 Methods for production of alginate coatings

4.1.1 Preparation of Sterile Alginate

The alginate electrode coatings developed in this project are intended for in-vivo application. Therefore there is a need for the preparation of sterile alginate. This is essential at this preliminary stage, even though in vivo work is not involved, to take into account any changes to the polymer composition and structure that may result from this sterilization process. Sterile alginate was prepared using two different methods (i) filtration and (ii) autoclaving.

Alginate powder was purchased from FMC Biopolymer (Drammen, Norway) (LF200M, Batch# S15596). The alginate had a G-block to M-block ratio of 35-45:55-65. The viscosity of 1% alginate solution prepared at 20°C is 200-400 mPa as per manufacturer's description.

For preparing sterile alginate by filtration, a 1% (w/v) alginate solution was prepared by mixing small proportions of the alginate powder with distilled water until a homogenous solution was obtained. The solution was then filtered using a 0.45 μm Nalgene sterile syringe filter. A 1% (w/v) alginate solution was chosen because it was much harder to filter higher concentrations of alginate using syringe filters. The filtered 1% alginate solution was then placed in 50 ml tubes and instantaneously frozen using liquid nitrogen. The frozen 50 ml tubes were then placed in a -80° C freezer for 3-4 hours. After ensuring complete

freezing of the alginate, the solution was placed in a freeze drying apparatus for 1-3 days. The freeze dried alginate was weighed using a scale to ensure complete loss of moisture. A 4% (w/v) alginate solution was then prepared using the freeze dried alginate by mixing with distilled water.

To prepare autoclaved alginate, a 4% (w/v) alginate solution was prepared directly by mixing the alginate powder with distilled water. The 4% alginate solution was then autoclaved at 121°C for 35-45 minutes to obtain sterile alginate. The autoclaved 4% alginate solution and the filtered 4% alginate solution were visually inspected for changes in viscosity and their ability to form crosslinked gels.

4.1.2 Protein Solubility Test

The protein solubility test was performed to determine the effect of dissolving high pI proteins like BDNF in filtered 4% alginate solution. Due to the high cost of BDNF all experiments were carried out using model proteins with similar properties (molecular weight and pI) (Table 4.1).

Lysozyme, α-chymotrypsin, and Bovine Serum Albumin (BSA) were obtained from Sigma-Aldrich (Saint Louis, MO). Chymotrypsinogen-A was purchased from Worthington Biochemical Corp. (Lakewood, NJ). A 8mg/ml solution of all proteins was prepared by dissolving the lyophilized powders of the different proteins in distilled water. The solubility of the different proteins in 4% alginate was then tested by mixing 500µl-1ml of the 8mg/ml protein solution

in alginate. Observations were made on the ability of the proteins to completely go into solution.

Table 4.1: Properties of different proteins used to test solubility in alginate

Protein	Isoelectric point	Molecular weight (Da)
Brain Derived Neurotrophic factor (BDNF)	9.01	27,200
Bovine Serum Albumin (BSA)	4.7	66,000
Chymotrypsin	9.1	25,000
Lysozyme	11.35	14,700
Chymotrypsinogen	9.1	25,000

4.1.3 Cleaning Silicon wafers

Silicon wafers ~500 μms thick were purchased from Silicon, Inc. (Kuna, Idaho). The purchased silicon wafers were cut using a diamond cutter or broken using a spatula to form 2cm x 2cm squares. The broken wafers were then cleaned to remove any organic impurities using the two step RCA (Radio corporation of America) cleaning process described below (Kern, 1990). All solutions for the cleaning process were purchased from Sigma-Aldrich (Saint Louis, MO).

For the first cleaning step the wafers were placed on a holder and immersed into a beaker containing 250 ml of distilled water. To the distilled water 50 ml of 28% ammonium hydroxide solution (NH_4OH) and 50 ml of 30% hydrogen peroxide (H_2O_2) were added in such a way that the ratio of water, NH_4OH and H_2O_2 was 5:1:1. The beaker containing wafers and solution was then placed on a hot plate and allowed to boil for 5-10 minutes. The temperature

of the solution was under constant check and was not allowed to exceed 72°C. This was important to prevent the exothermic reaction from going out of control. When finished, the wafer was transferred to a container with overflowing distilled water. After 4-6 changes of water, the wafers were removed under flowing water to avoid any organic residue from re-depositing on the wafer surface.

For the second cleaning step the same procedure as the one mentioned above was used. However to the 250 ml of distilled water, 50 ml of concentrated hydrochloric acid (conc. HCL) and 50 ml of 30% hydrogen peroxide (H_2O_2) were added in a volume ratio of 5:1:1. The solution with wafers was then allowed to boil for 5-10 mins before washing it again with 4-6 changes of water.

After the two step cleaning process the wafers were allowed to dry overnight and wiped clean the next day. All washed wafers were stored in a closed dry chamber until ready to be used for experiments.

4.1.4 Spinner coating parameters testing

To test the effect of alginate concentration, filtered 2% and 4% alginate solutions were prepared as mentioned above. Using a 1 ml syringe the 2% or 4% alginate solutions were applied on the surface of clean silicon wafers. Thin films of alginate were then produced by spin coating the silicon wafers in a WS-400B-GNPP/LITE/AS spin processor (Laurell Technologies, North Wales, PA). To compare the effect of processing parameters, alginate was spin coated at different

operating velocities of 1000, 2000 and 3000 rpm for different durations of 30 and 60 seconds.

Following spinner coating, the alginate coatings were crosslinked by spraying a 10% solution of $CaCl_2$ on the surface of the wafers.

4.1.5 Crosslinking of alginate coatings

Two different systems of crosslinking were tested for producing alginate coatings (i) fast $CaCl_2$ crosslinking or (ii) slow $CaCO_3$+GDL (D-glucono-□ lactone) crosslinking. For fast crosslinking a 10% $CaCl_2$ was sprayed on the surface of the alginate (figure - 4.1). A total of about ~300µl (3-4 sprays from a 3 ounce spray bottle) of 10% $CaCl_2$ was sprayed using a spray bottle on the surface of the alginate coatings.

For the slow $CaCO_3$+GDL crosslinking, 1ml of filtered 4% alginate solution was mixed with 100 µl of 150 mM $CaCO_3$ suspension and 100 µl of 150 mM GDL (6-7 hour old). Experiments performed in the lab showed that fresh GDL took a very long time to crosslink the alginate gels. However when GDL was allowed to hydrolyze for 6-7 hours, crosslinking of alginate occurred within a period of 1-1.5 hours following mixing. The solution of alginate, GDL and $CaCO_3$ was then spin coated on silicon wafers using the same method described above (Figure 4.1).

Following crosslinking, all wafers with alginate were placed on a hot plate at the lowest temperature setting (30-40°C) until the coatings were moderately dry. Care was taken not to completely dry (visualized by the formation of air pockets or cracks on the alginate surface) the coatings, since it resulted in the formation of cracks. Thickness measurements of the alginate coatings were obtained using a profilometer (Zygo Corp., Middlefield, CT).

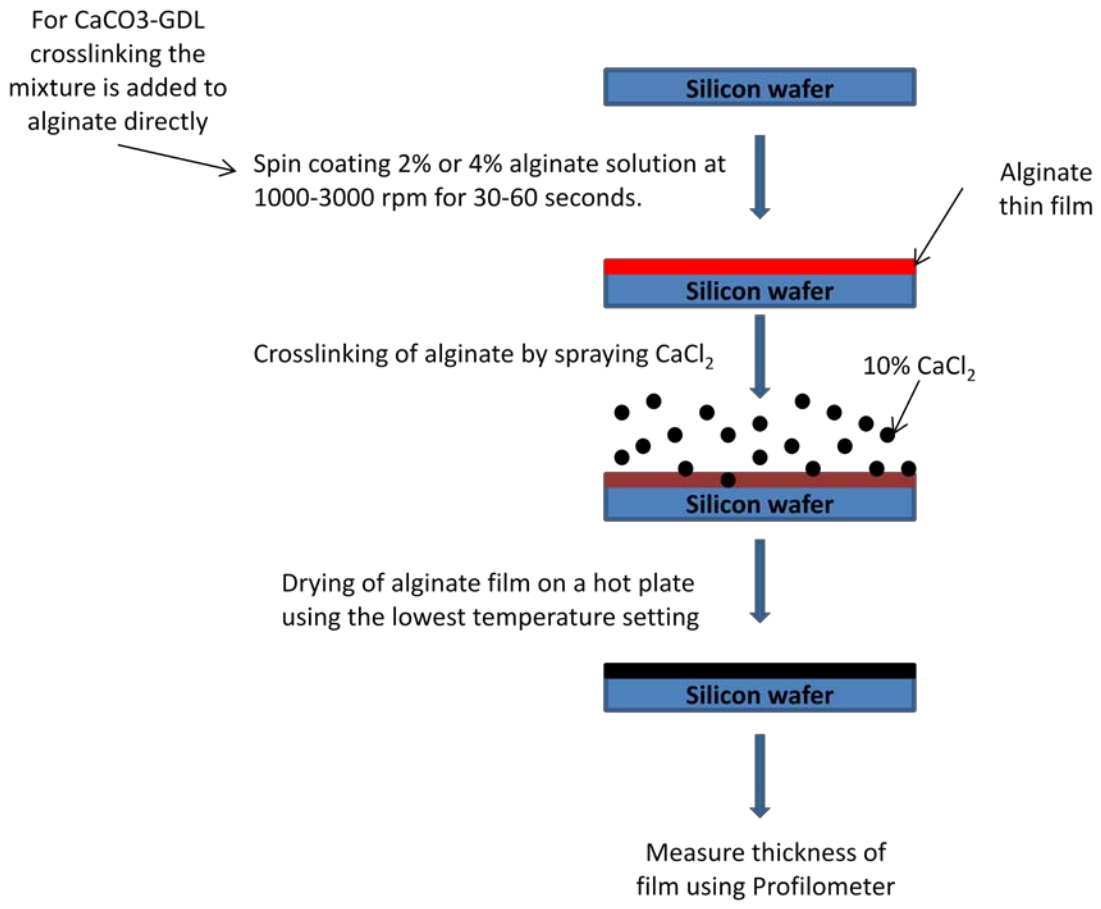


Figure 4.1: Step-by-Step procedure for producing crosslinked alginate thin films using spinner coating

4.1.6 Production of multilayered alginate coatings

To determine the feasibility of manufacturing thicker alginate coatings, spinner coating of multiple layers of alginate was performed. Four different wafers (three replicates for each type) with one, two, three or four coatings of alginate were spin coated using the spin coater (Figure 4.2).

A solution of filtered 4% alginate was used for all experiments. The alginate solution was spin coated on silicon wafers at 1000 rpm for 30 seconds to obtain an even coating. The coating was then dried by placing the silicon wafer on a hot plate using the lowest temperature setting. Once dry, the wafer was again spin coated using 4% alginate at 1000 rpm for 30 seconds to obtain a second coating. The third and fourth coatings were applied using the same method.

All wafers were finally crosslinked using a 10% solution of $CaCl_2$ (3-4 sprays from a 3 ounce spray bottle) and subsequently placed inside a humidified chamber (100% humidity) overnight. Thickness measurements of the alginate coatings were then obtained using a profilometer (Zygo Corp., Middlefield, CT).

4.2 METHODS RELATED TO TESTING

4.2.1 Measuring thickness of alginate coatings using Profilometer

To measure the thickness of the alginate coatings an optical white light profilometer was used (Zygo Corp., Middlefield, CT). Following crosslinking an

artificial step was scratched in each film using a razor blade and the height difference between the film surface and the underlying silicon substrate was measured at 4 nearby points in the region of the scratch.

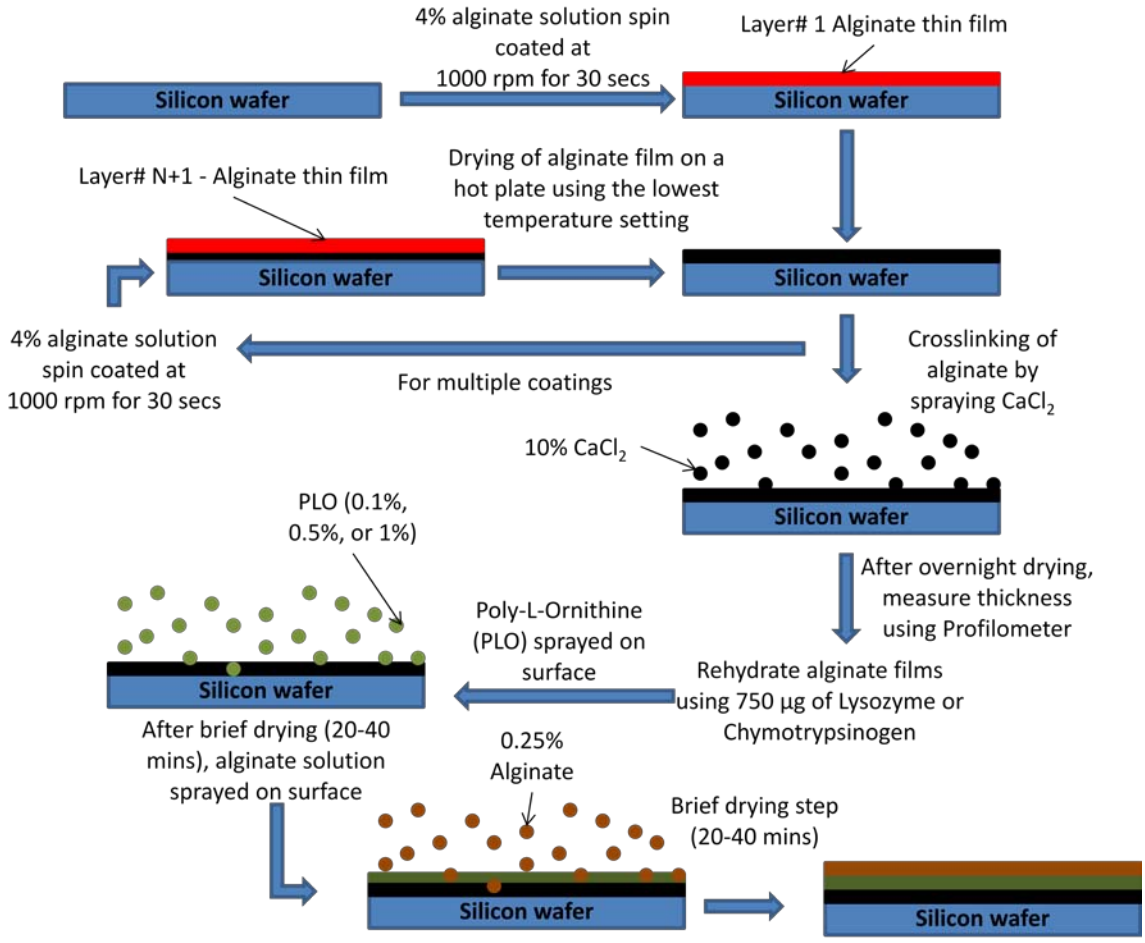


Figure 4.2: Step-by-Step procedure for producing multilayered alginate films coated with Poly-L-ornithine (PLO)

4.2.2 Protein loading into alginate coatings:

Traditionally protein loading into alginate beads has been performed either by (i) mixing the protein with the alginate solution prior to crosslinking or

(ii) by diffusing the protein into alginate coatings or beads after crosslinking. The former method of protein loading was not used due to strong alginate-protein interactions (Wells and Sheardown, 2007).

Therefore all proteins were diffused into the alginate coatings once they were crosslinked with 10% $CaCl_2$. Once again two different methods were identified for protein loading by diffusion. To test both methods, two groups of three layered films manufactured using 4% filtered alginate solution was used (figure 4.2).

The first group of dry alginate coatings was immersed into a beaker containing 10 ml of 4 mg/ml protein solution. The second group of dry alginate coatings was allowed to re-hydrate with 150-175 μl of either lysozyme or chymotrypsinogen to obtain a final loading of 750 μg of protein in each film (figure 4.2).

4.2.3 Poloxamer (p-188) loading into alginate coatings

To check the ability of the coatings to deliver poloxamer (p-188) was loaded into the alginate coatings. To track the release of the poloxamer from the alginate coatings, the poloxamer was fluorescently labeled with fluorescein isothiocyanate (FITC) to prepare FITC-p-188. The FITC-p-188 was loaded into the alginate coatings by rehydration, similar to protein loading. Dried alginate coatings prepared by spin coating were rehydrated using 150-175 μl of FITC-p188 to obtain a final loading of 750 μg of poloxamer.

4.2.4 PLO coating of alginate

To prolong protein release from alginate coatings the use of poly-L-ornithine (PLO) coatings was evaluated. All PLO coated wafers containing protein/p-188 were used for release studies almost immediately. Poly-L-ornithine hydrochloride, molecular weight 15,000 – 30,000 Da was purchased from Sigma Aldrich (St. Louis, MO).

Three different concentrations of PLO 0.1% (w/v), 0.5% or 1% were prepared in HEPES buffer (pH 7.4). Once prepared the different PLO solutions were sprayed on the surface of crosslinked alginate films (3-4 sprays from a 3 ounce spray bottle). The wafers were then allowed to dry for 20-40 minutes at room temperature. During this time care was taken to prevent complete drying of all wafers. Complete drying of alginate coatings often produced cracks on the surface, making them unusable for subsequent release studies.

Irrespective of the concentration of the PLO used all wafers were coated with a final layer of alginate. This was achieved by spraying the already PLO coated wafers with a 0.45 μm filtered 0.25% (w/v) alginate solution. The wafers were then allowed to dry again for 20-40 minutes. Figure 4.3 shows the schematic of the different coatings on the silicon wafers.

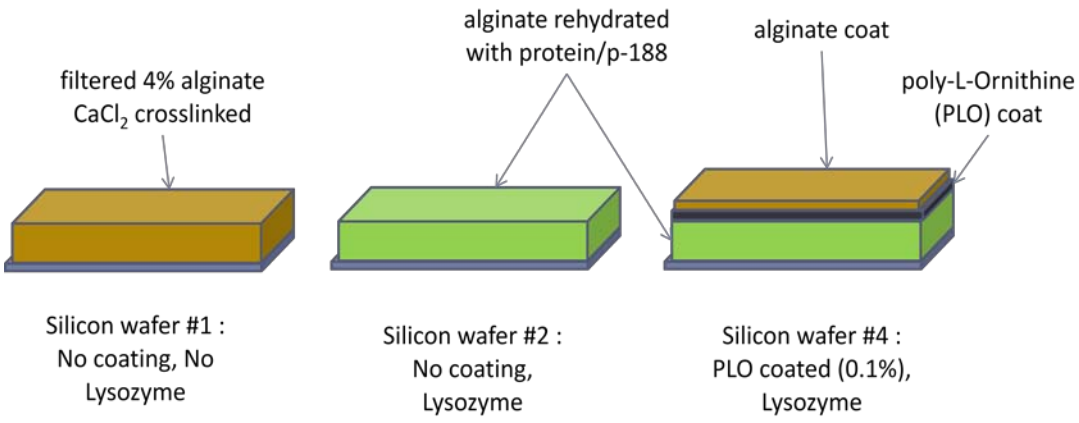


Figure 4.3: Schematic of spin coated wafers with alginate-PLO-alginate coatings

4.2.5 Release studies

For the release study all treatment wafers were re-hydrated with 150-175 µl of either lysozyme or chymotrypsinogen or poloxamer (p-188) to obtain a final loading of 750 µg of protein/p-188 in each wafer (Figure 4.2). All control wafers were re-hydrated with an equal volume of distilled water. Table 4.2 shows the different control and treatment wafers used for the release studies.

Release studies were conducted at 37 °C in beakers containing 5 ml of 1x PBS. The beakers containing PBS were then placed on an orbital shaker set at 20 rpm.

Table 4.2: Treatments and controls used for testing protein/p-188 release from alginate coatings

Silicon wafer#	Coating	Neuroprotective drug
Wafer #1	-	-
Wafer #2	-	Lysozyme
Wafer #3	PLO coat (1%)	-
Wafer #4	PLO coat (0.1%)	Lysozyme
Wafer #5	PLO coat (0.5%)	Lysozyme
Wafer #6	PLO coat (1%)	Lysozyme
Wafer #7	PLO coat (0.1%)	Chymotrypsinogen
Wafer #8	PLO coat (0.5%)	Chymotrypsinogen
Wafer #9	PLO coat (1%)	Chymotrypsinogen
Wafer #10	-	FITC-p-188
Wafer #11	PLO coat (0.1%)	FITC-p-188
Wafer #12	PLO coat (0.5%)	FITC-p-188
Wafer #13	PLO coat (1%)	FITC-p-188

At each time point the entire 5 ml of 1xPBS was removed and replaced with fresh solution. The samples from each time point were then separated into two groups by placing them in two separate microcentrifuge tubes as shown in Figure 4.4. The first group of samples underwent centrifugation at 3000 rpm for 1 min to separate the protein solution from particulate matter. In comparison, to the second group of samples, 100 μl of 4mg/ml EDTA was added and mixed. Following mixing the second group of samples also underwent centrifugation at 3000 rpm for 1 min. The concentration of total dissolved protein in each group was then determined to obtain the final release profile.

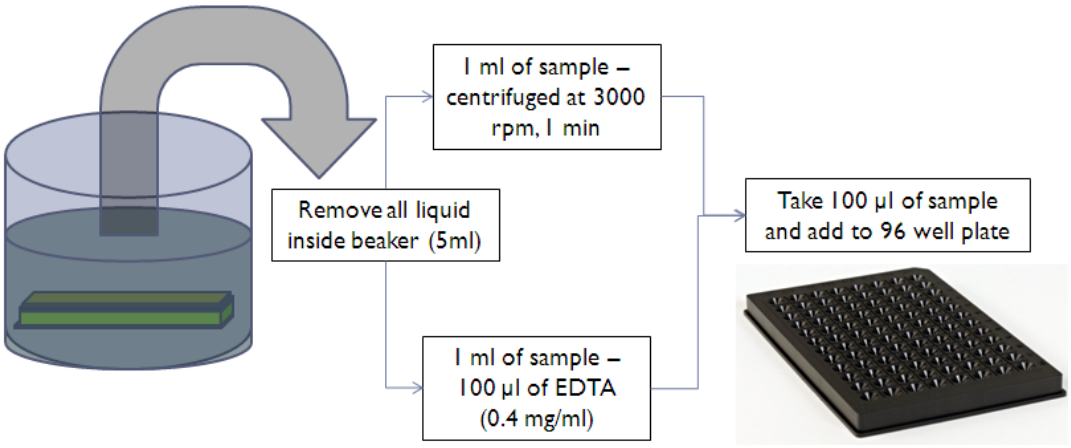


Figure 4.4: Schematic of release study showing sample isolation and protein quantification

4.2.6 Protein Quantification

All samples obtained from release studies were immediately assayed for protein concentrations or were stored in 4°C refrigerators up to a maximum of 4 days. Protein quantification was performed using the FluoroProfile™ Protein Quantification Kit (Sigma cat no. FP0010). The assay was carried out in a 96 well plate format using Corning black 96 well plates with flat bottoms (Coring no: 3915). A standard curve was prepared for each protein using known concentrations of protein dissolved in 1xPBS or sample buffer. Using the standard curve unknown concentrations of proteins were calculated by interpolation.

For the protein assay either 50 µl or 100 µl of sample were used. Depending on the volume of sample, an equal volume of protein assay buffer (50 µl or 100 µl) was added to each well of the 96 well plate. Care was taken to not create any bubbles in the process. Once the samples and assay buffer were added to the respective wells, the plates were then placed on an orbital shaker (20 rpm) in the dark for up to 1 hour. Fluorescence quantification from the plates was measured using a TECAN microplate reader at an excitation wavelength of ~510 nm and emission wavelength of ~620 nm.

4.2.5 Poloxamer quantification:

Quantification of poloxamer was possible due to the FITC conjugated to it and was performed almost immediately after the release study. Similar to the protein quantification mentioned above, standard curves were constructed using known quantities of p-188. Using the standard curve, unknown concentrations of p-188 at the different time points were calculated by interpolation. Quantification of the FITC-p-188 was done using a TECAN reader at an excitation wavelength of ~485 nm and emission wavelength of ~525 nm.

5 RESULTS AND DISCUSSION

The main goal of this project was to promote long term in-vivo functionality of silicon electrodes by delivering BDNF from alginate electrode coatings. The overall project consisted of two main phases. During the first phase of the project, experiments were performed using the spinner coating apparatus. Results from these experiments were used to identify the right combination of material and methodological parameters needed to manufacture electrode coatings of the required thickness.

During the second phase of the project, release studies were performed from alginate coatings manufactured using the parameters obtained from the first phase. The release of two model proteins (lysozyme and chymotrypsinogen) and FITC labeled poloxamer (FITC-p-188) was performed in phosphate buffered saline (PBS) at 37°C. Results from the release studies showed the release profiles of the two model proteins and poloxamer over a period of 48 hours. Finally the effect of Poly-L-ornithine (PLO) coatings to provide controlled release was evaluated.

5.1 Manufacturing alginate thin films

5.1.1 Determining optimal sterilization technique

In the past sterile alginate has been prepared either by autoclaving or by filtering. However, depending on the sterilization method used the physical properties of the alginate have been shown to vary. Therefore choosing the right method of sterilization for this specific application will be important.

Results from the sterilization experiments showed that autoclaved 4% alginate solutions had a much lower viscosity compared to the filtered 4% alginate solutions. These observations are in accord with studies which report autoclaving alginate (Leo W. J., 1990). Autoclaving alginate has been shown to cause a decrease in the degree of polymerization of the alginate molecules (Leo W. J., 1990). Leo et al., studied the effect of sterilization on the physical properties of alginate. The results of the study showed that 1% and 3% solutions of alginate when autoclaved at 121°C for 20 minutes measured a 78% and 86% decrease in viscosity respectively (Leo W. J., 1990).

The viscosity of the alginate solution is an important variable that needs to be considered for producing alginate coatings via spinner coating. Highly viscous solutions of alginate are expected to produce thicker coatings while solutions of low viscosity are expected to produce relatively thinner coatings. The results from the spinner coating experiments showed that 4% autoclaved alginate solutions spin coated at 1000 rpm for 30 seconds ($CaCl_2$ crosslinked) produced coatings in the range of nanometers. However spin coating of 4% filtered alginate solution using the same parameters produced coatings in the lower microns (~5-6 μm). Also the coatings formed using the autoclaved alginate were brittle and developed cracks easily compared to the coatings manufactured using the filtered alginate solutions.

Based on the thickness measurements either 4% autoclaved alginate or 4% filtered alginate could be used to produce electrode coatings. However to

reach a final coating thickness of 20-30 μm fewer layers of filtered 4% alginate solution would be required. Therefore filtering alginate was chosen as the preferred method of sterilization.

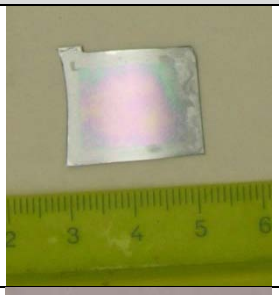
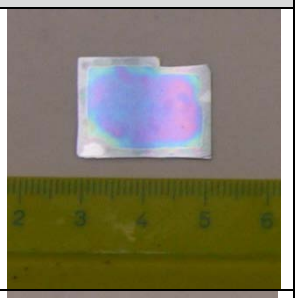
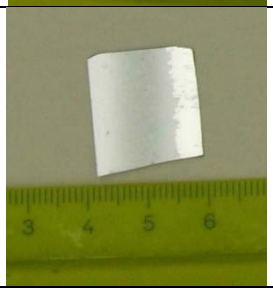
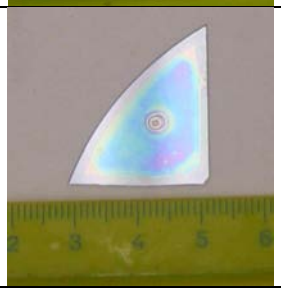
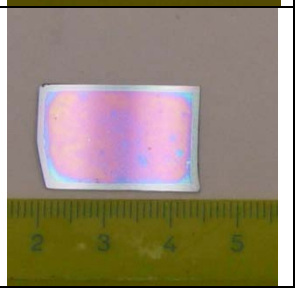
5.1.2 Effect of alginate concentration, velocity of rotation and spin coat duration

Optimization of processing variables (spin-coating velocity, acceleration and duration) and variables associated with the polymer solution (concentration, molecular weight, pH, temperature etc.) is essential for consistent alginate coating production. Among the variables associated with the polymer solution all were kept constant except for the concentration of the solution. To check the effect of alginate concentration, solutions of two different concentrations, 2% (Table 5.1) and 4% (Table 5.2) were used for manufacturing electrode coatings. While both concentrations of alginate produced electrode coatings, a wide variation in thickness was observed. As seen from Table 5.1 the coatings manufactured using the 2% alginate solutions were much more colorful compared to the coatings manufactured using 4% alginate solutions (Table 5.2). The variation in color between the two different coatings can be attributed to the variation in the thicknesses of the films. Thickness variation in the films results in variation in the film's surface properties by the creation of phase changes in the angle of refraction. The equation relating phase changes with the film's thicknesses is: $\mathbf{\Phi\lambda = nd}$ where Φ is the phase change, λ is the wavelength, n is the index of refraction of the film, and d is the thickness of the film.

Therefore based on the reflective properties of alginate coatings, colorful surfaces would imply thinner coatings while colorless surfaces implied comparatively thicker coatings. Therefore films manufactured using the 2% alginate solution were a lot thinner compared to the films manufactured using the 4% alginate solution.

The above observations confirmed initial expectations, since 4% alginate solution being highly viscous, was expected to produce comparatively thicker coatings. The results from the profilometer studies further confirmed the above observations by showing that coatings manufactured using 4% alginate solution (1000 rpm, 30 secs, $CaCl_2$ crosslinked) were thicker by 2-3μm compared to coatings manufactured using 2% alginate solutions (1000 rpm, 30 secs, $CaCl_2$ crosslinked).

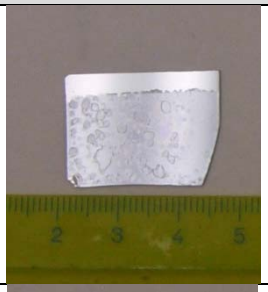
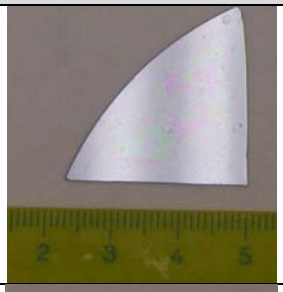
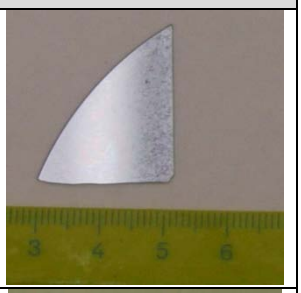
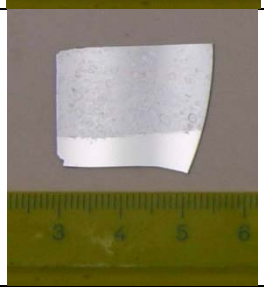
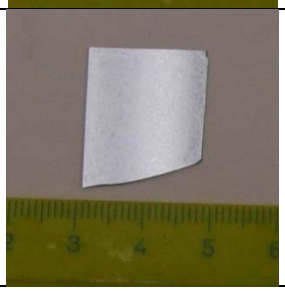
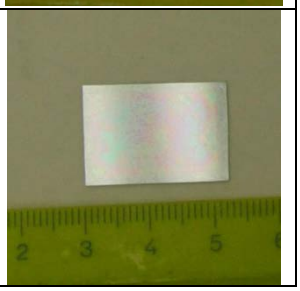
Table 5.1: Thickness and reflectance properties of alginate coatings spin coated using 2% filtered alginate solution and crosslinked with $CaCl_2$

2 % Alginate solution ($CaCl_2$ crosslinked)		Velocity of Rotation (rpm)		
		1000	2000	3000
Duration of Spinning (sec)	30 seconds			
	60 seconds			

Among the different processing variables spin coating velocity and duration of spinning were changed keeping all else constant. Both the variables had a similar effect on the thickness of the alginate coatings. When the spin coating velocity was increased it resulted in the production of more colorful and hence thinner coatings. This property although not very obvious with the coatings manufactured using 4% alginate solutions (Table 5.2), is evident with the coatings manufactured using the 2% alginate solutions (Table 5.1). As seen from Table 5.1 a clear variation in thickness is noticed via the reflective properties of the coatings when the speed is increased from 1000 rpm to 3000

rpm. The coatings spun at 3000 rpm are colorful and a lot thinner compared to coatings spun at 1000 rpm.

Table 5.2: Thickness and reflectance properties of alginate coatings spin coated using 4% filtered alginate solution and crosslinked with $CaCl_2$

4 % Alginate solution ($CaCl_2$ crosslinked)		Velocity of Rotation (rpm)		
		1000	2000	3000
Duration of Spinning (sec)	30 seconds			
	60 seconds			

Increasing the duration of spinning produced a very similar effect. With increasing duration a decrease in thickness of the coatings was observed. Comparison of alginate coatings (4% alginate solution, 3000 rpm) spun for 30 and 60 seconds shows a much colorful and hence thinner coating produced by the latter compared to the former (Table 5.2). Thickness measurements obtained from the profilometer confirmed the above observations. The results from the profilometer showed that the thickest coating was manufactured using the 4%

alginate solution spun at 1000 rpm for 30 seconds (~6 μm). When the duration of rotation was increased to 60 seconds the thickness decreased to ~5 μm. Similarly when the spin coating velocity was increased to 2000 rpm or 3000 rpm the thickness of the coatings manufactured decreased to the range of nanometers.

Since coatings in the range of microns are required the parameters that produced the thickest film (4% alginate solution, 1000 rpm, 30 seconds) was used subsequently for manufacturing alginate coatings. The parameters were chosen in the interest of requiring fewer layers of alginate to reach the final coating thickness.

5.1.3 Slow $CaCO_3$ crosslinking versus fast $CaCl_2$ crosslinking

Degradation of non-crosslinked plain alginate coatings in 1xPBS was found to be in the order of seconds (results not shown). Therefore to prolong the degradation time of the alginate coatings in 1xPBS crosslinking using divalent cations was necessary.

In the past crosslinking of alginate has been done using a number of different methods. For this specific project two different methods of crosslinking alginate were evaluated (i) the slow gelling $CaCO_3$ + GDL method and (ii) the fast crosslinking method using $CaCl_2$.

Table 5.3: Alginate coatings manufactured using 2% alginate crosslinked using slow gelling $CaCO_3$ –GDL method

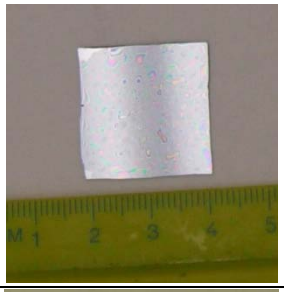
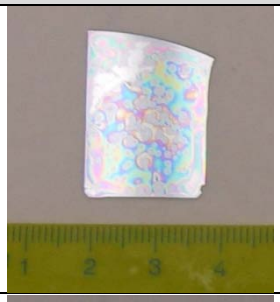
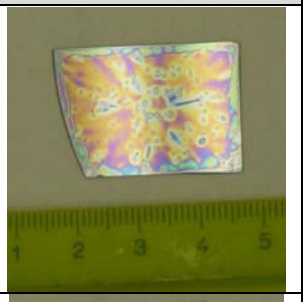
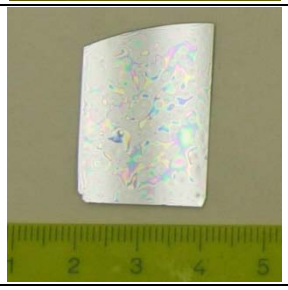
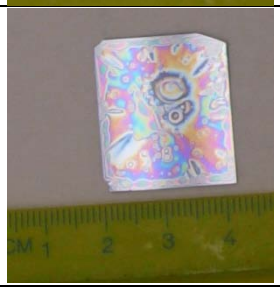
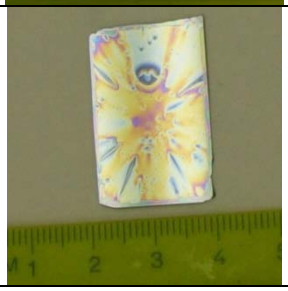
2 % Alginate solution ($CaCO_3$+GDL crosslinked)		Speed of Rotation (rpm)		
		1000	2000	3000
Duration of Spinning (sec)	30 seconds			
	60 seconds			

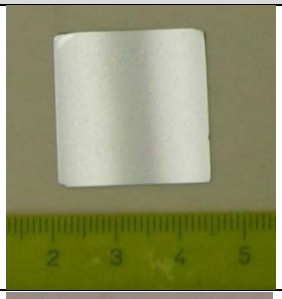
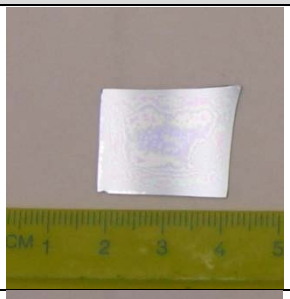
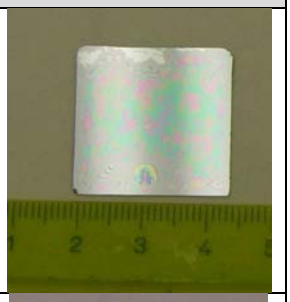
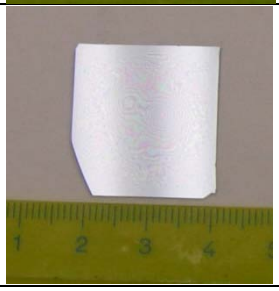
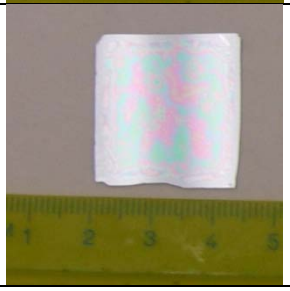
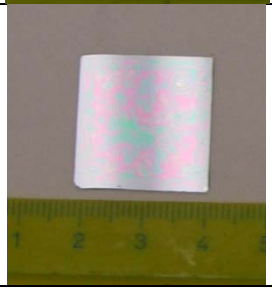
Table 5.1- Table **5.2** and Table **5.3**-Table **5.4** show the results obtained after crosslinking alginate using the fast crosslinking and the slow crosslinking methods respectively. Comparison of the coatings manufactured using these two methods shows very little difference. As seen from the tables, both methods produced alginate coatings with colorful surfaces at high velocities (>1000rpm). Similarly coatings with colorful surfaces are observed when the duration of spinning was increased from 30 to 60 seconds. The only major difference noticed between the two crosslinking methods was with regards to the surface profile of

the alginate films. The surface profile of coatings manufactured using the slow gelling method was relatively uneven. This was due to the presence of insoluble $CaCO_3$ crystals found on the surface. Efforts to achieve complete solubility failed due to the fact that the rate of $Ca2^+$ liberation due to hydrolyzing GDL was much slower than the rate of drying of the film. Results from the crosslinking experiments showed that complete solubility and subsequent crosslinking took up to 8 hours following addition of fresh GDL. During this time the alginate films most often dried leaving un-dissolved $CaCO_3$ crystals on the surface of the wafers. Table 5.3 shows the unevenness of the crosslinking seen in the form of dots on the surface of the alginate films. In comparison using an aerosolized spray of $CaCl_2$ produced even crosslinking. The aerosolized spray of calcium chloride solution delivered the Ca2+ ions to the films without disturbing the surface structure. This can be seen by the even coloration on the surface of the thin films as seen in Table 5.1 and Table 5.2.

Cathell et al., showed that aerosolized spray of $CaCl_2$ is the optimal method for crosslinking alginate thin films (Cathell and Schauer, 2007). The authors of the study did not evaluate the slow-gelling method. However they conducted a number of studies to decipher the optimal method of crosslinking using $CaCl_2$. Experiments were conducted where (i) $CaCl_2$ was added to the alginate solution right before spin coating or (ii) $CaCl_2$ was added using an automated dispensing mechanism to the thin films during the spin coating

process or (iii) thin films were immersed into solutions of $CaCl_2$ right after spin coating or (iv) $CaCl_2$ was added in the form of an aerosolized spray (Cathell and Schauer, 2007). Results from these studies showed that the first three experiments failed to produce even crosslinked alginate films. The first two experiments failed as they led to formation of rapidly crosslinked insoluble gels before alginate thin films could be manufactured. The third experiment failed since it often led to the disruption of the alginate films when immersed in a solution of $CaCl_2$. The aerosolized spray of $CaCl_2$ was found to be the optimal method for crosslinking films produced via spinner coating.

Table 5.4: Alginate coatings manufactured using 4% alginate crosslinked using slow gelling $CaCO_3$–GDL method

4 % Alginate solution ($CaCO_3$+GDL crosslinked)		Speed of Rotation (rpm)		
		1000	2000	3000
Duration of Spinning (sec)	30 seconds			
	60 seconds			

5.1.4 Characterization of multilayered alginate thin films

Based on observation, coatings measuring approximately 6 μm of thickness were produced by spin coating 4% filtered alginate at 1000rpm for 30 seconds (Figure 5.1). Release studies performed in saline using the alginate coatings showed that the films degraded completely within a matter of minutes. Therefore to increase degradation times, thicker films manufactured using multiple coats (layers) of alginate was required.

To prepare thicker films 1, 2, 3 and 4 coatings of alginate was performed on a single silicon wafer using the method shown in Figure 4.2. Thickness measurements obtained using the profilometer showed that films ranging from 5-80 μm in thickness could be manufactured by increasing the number of alginate layers. The average thickness recorded for the 1, 2, 3 and 4 alginate coatings was 6.14 μm, 26.16 μm, 46.53 μm and 80.25 μm respectively (Figure 5.1). Although promising, increasing thickness using multiple coatings also increased surface unevenness. Looking at Figure 5.1 we see that the errors associated with film thicknesses increased with increase in the number of coatings. The film manufactured using 4 coatings recorded the maximum standard error of 9.12 μm while the film manufactured using a single coating recorded the least error of 0.37 μm.

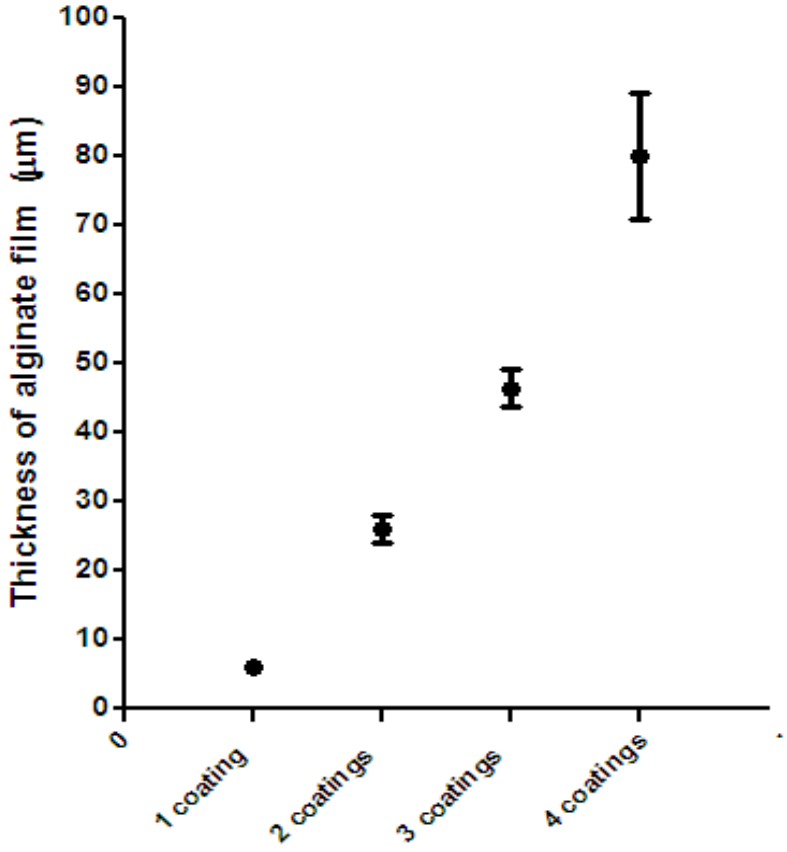


Figure 5.1: Change in thickness with number of alginate coatings (Alginate 4%, 0.45 µm filtered, 10% $CaCl_2$ crosslinked)

When compared to the single coated films, films manufactured using 4 coatings took several hours to degrade in 1xPBS at 37°C. However they weren't used since they exceeded the initial design requirement for a coating that measured less than 50 µm. In comparison films manufactured using 2 or 3 alginate coatings fit the initial design requirement, they recorded very little error in their thickness profiles, 2.02 µm and 2.64 µm respectively. Experiments on protein loading showed that films manufactured using 2 and 3 coatings could be loaded with physiologically relevant quantities of protein.

Based on the results either 2 or 3 layered alginate coatings could have been used for subsequent protein release studies. However the 3 layered alginate film was chosen over the 2 layered film since they were expected to degrade over a longer period, hence sustaining protein release for a longer period of time.

5.1.5 Protein-alginate interactions

The results from the protein solubility tests showed that proteins with high isoelectric points (pI > 7) formed precipitates when mixed with 4% filtered alginate. On the other hand low pI proteins (pI < 7) like BSA completely dissolved in the alginate solutions (Table 5.5). Although all three high pI proteins (Lysozyme- pI – 11.35, α-Chymotrypsin- pI – 9.1 and Chymotrypsinogen A- pI – 9.1) formed precipitates at a final concentration of 4mg/ml, very strong protein-alginate interactions were observed only in lysozyme. The protein-alginate interaction seemed comparatively weaker in chymotrypsin and chymotrypsinogen. When the final protein concentration was reduced to 2mg/ml both chymotrypsin and chymotrypsinogen dissolved in alginate with vigorous mixing. However lysozyme (pI – 11.35) continued to form a thick white precipitate even at a lower concentration of 2mg/ml.

The above observations are concurrent with the results of Wells et al. (Wells and Sheardown, 2007). Wells et al., tried to encapsulate high pI proteins (Lysozyme and Chymotrypsin) and low pI proteins (BSA) in 3% alginate solutions to form microspheres. The results of the study showed 1 ml of 30

mg/ml solutions of both lysozyme and chymotrypsin interacted with 3% alginate solution forming a precipitate (Wells and Sheardown, 2007). On the other hand BSA completely dissolved in the alginate solution up to a concentration of 15mg/ml (Wells and Sheardown, 2007). While Wells et al., report precipitation with high pI proteins, Vögelin et al. reports successful solubility of BDNF (pI 9.1). The study reports successful mixing of 600 μg of BDNF with 1 ml of 3% sodium alginate solution without any signs of precipitation (Vögelin et al., 2006).

Table 5.5: Solubility of high and low pI proteins in 4% filtered alginate solution

Protein	Isoelectric point (pI)	Size of protein (kDa)	Solubility
Bovine Serum Albumin (BSA)	4.7	66	soluble
Lysozyme	11.35	14.7	precipitate formed
Chymotrypsin	9.1	25	precipitate formed
Chymotrypsinogen	9.1	25.6	precipitate formed
Brain Derived Neurotrophic Factor (BDNF)	9.1	27.2	N/A

Taking into consideration the results from the solubility test and from other studies, it is clear that a high pI protein like BDNF will become electropositive at pH up to their pI and hence react with the negative carboxyl groups of the alginate forming a precipitate at high concentrations. It could be possible to overcome this problem by using a lower concentration of BDNF. However the lower concentration will create problems in achieving loading of physiologically relevant quantities of BDNF to promote neuron survival.

5.2 Protein release studies

5.2.1 Optimal method for loading proteins into alginate films

Results from the solubility test clearly showed that loading of proteins by mixing with alginate solutions is not feasible. Therefore two different methods for loading proteins were tested. Proteins were loaded either by (i) immersing the dry alginate coatings into a protein solution, or (ii) by rehydrating the films using a pipette containing 100-175 μl of concentrated protein solution.

Comparison of the two methods showed that the latter led to more efficient protein loadings compared to the former. Dry alginate coatings when immersed into a protein solution rehydrated, expanded rapidly and therefore detached from the underlying silicon wafers making them unusable for subsequent protein release studies. In comparison the group of films that were rehydrated with 100-175 μl remained intact even after protein loading. Loading proteins by rehydrating using a pipette also ensured no wastage of protein during the loading process.

5.2.2 Protein Release Profiles – Lysozyme

The fluoroprofile protein kit was used to create standard curves using known quantities of lysozyme and chymotrypsinogen. Both the standard curves showed that protein concentrations ranging from 48.8 ng/ml to 200 μg/ml occurred over a linear profile (Appendix A). Also the R^2 values for both the curves were 0.99 showing the goodness of fit. Using interpolation within the

linear range, the unknown concentrations of proteins at the different time points were calculated. The calculated concentrations were then used to build the release profile for the different proteins. Figure 5.2 shows the release profile of lysozyme obtained using the above method.

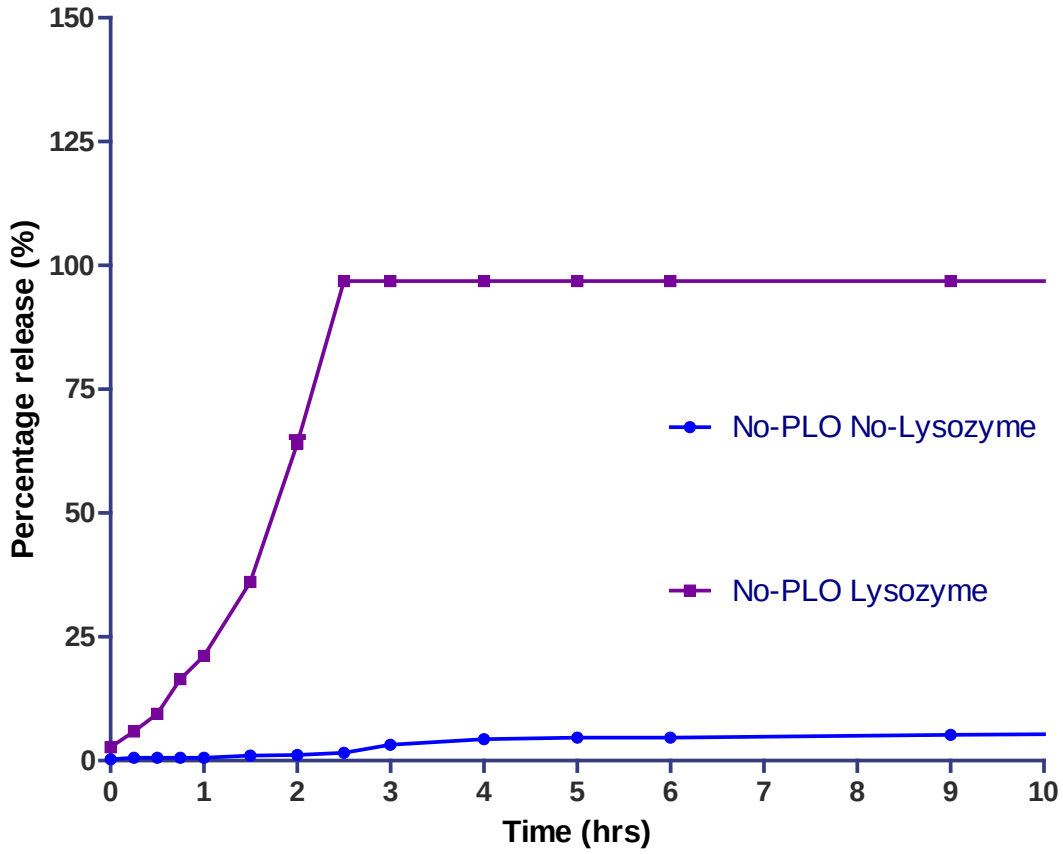


Figure 5.2: Lysozyme release from alginate thin films in 1xPBS, 37°C and 40 rpm

As seen from the release curve (Figure 5.2) the protein lysozyme starts exponential release immediately after it is placed in the PBS solution. Complete

release of the protein occurs within the first three hours, well short of the anticipated 3-4 day release. This three hour release is consistent with results obtained from other studies. Wells et al., showed that complete release of lysozyme in PBS occurred within three hours from alginate microbeads (Wells and Sheardown, 2007).

Looking past the three-hour time point the lysozyme release curve becomes flat indicating no more protein release. In comparison the control curve (No-Lysozyme) gradually increases starting at the 3 hour time point and reaches saturation at the 5-6 hour time point. This increase seen in the control curve is small and does not indicate the presence of any protein. Instead it is expected to be indicative of the degradation of the alginate in the release medium.

5.2.3 Effect of PLO Coating on Controlled Protein Release

Initial studies on protein release showed that complete release of proteins took place over a three hour period, well short of the anticipated period of 3-4 days. Therefore to prolong protein release, alginate thin films were coated with PLO. Previously, studies have shown that PLO can prolong protein release by (i) altering the network pore size of crosslinked alginate gels (Tobias et al., 2001) and/or (ii) increasing degradation time by forming polycation complexes with the alginate (Gombotz and Wee, 1998).

Figure 5.3 and Figure 5.4 show lysozyme release profiles obtained from alginate films coated with PLO. While the former shows the release profile over a

period of 48 hours the latter shows the release profile for the first 5 hours of protein release.

As seen in Figure 5.3, PLO-coated and uncoated films loaded with lysozyme have different release profiles compared to the negative controls (No-PLO No- Lysozyme and PLO (1%) No- Lysozyme). The negative controls do not contain any protein and therefore show zero release.

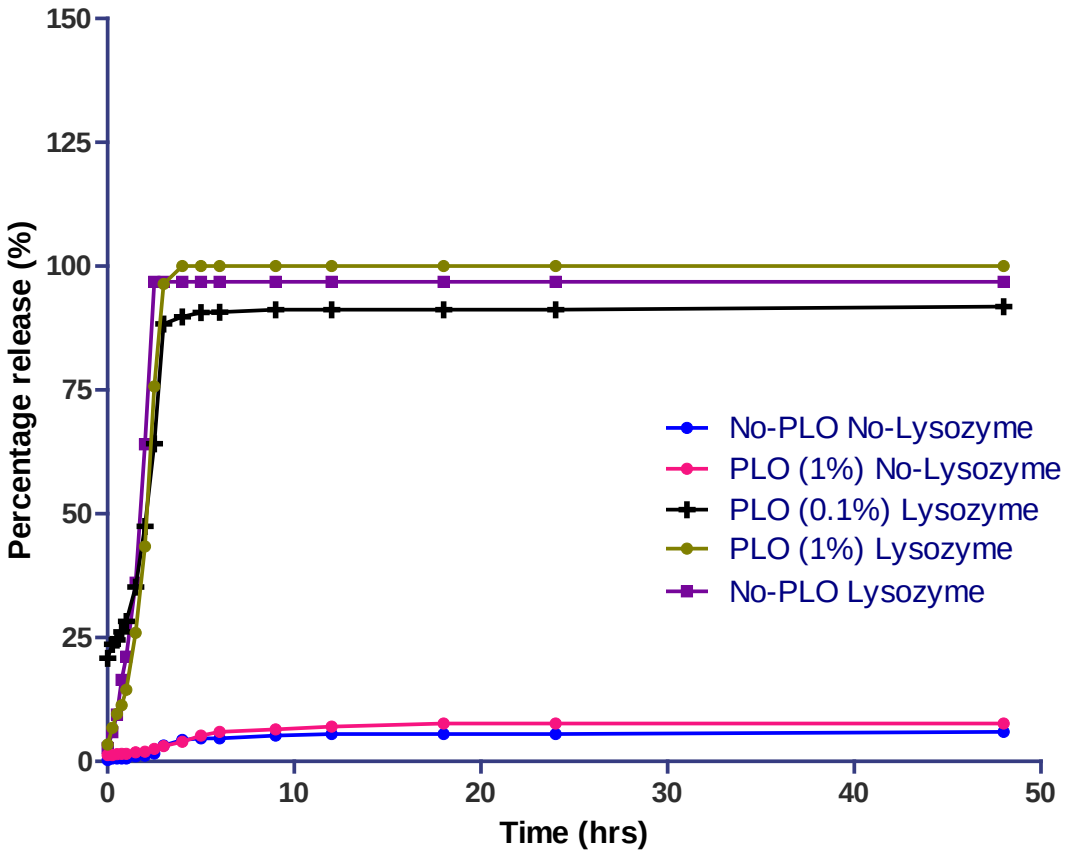


Figure 5.3: Lysozyme release from PLO coated alginate thin films over 48 hours

Comparison of PLO- coated and uncoated alginate films shows very little difference in their respective release profiles. As seen from Figure 5.3 protein release from alginate films coated with PLO (1%) and PLO (0.1%) resemble protein release from the uncoated alginate film (No-PLO Lysozyme). Therefore PLO coatings on alginate films had very little effect on prolonging protein release.

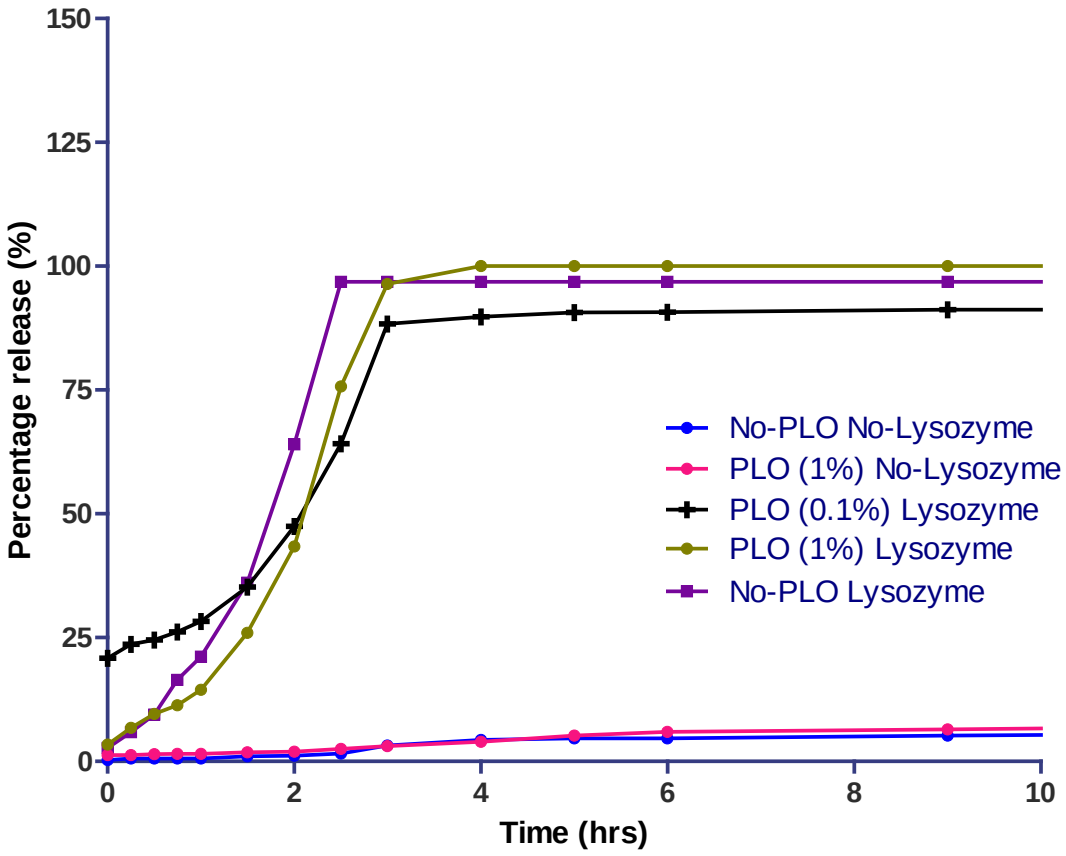


Figure 5.4: Lysozyme release from PLO coated alginate thin films – first five hours

Looking closely at the first 5 hours of lysozyme release (Figure 5.4) we see that both the uncoated (No-PLO Lysozyme) and coated films (PLO (1%) Lysozyme and PLO (0.1%) Lysozyme) show very similar release profiles. At time zero all the films show zero release save the film coated with 0.1% of PLO (PLO (0.1%) Lysozyme). The film coated with 0.1% PLO, releases approximately 25% of protein at time zero. This sudden release seen here is expected to be due to the detachment of the alginate film from the surface of the silicon wafer, resulting in protein diffusion from both sides of the film.

Release profiles of lysozyme and chymotrypsinogen from alginate films coated with different concentrations of PLO showed very little difference. It was expected that increasing the PLO concentration would result in smaller alginate network pores and/or thicker coatings on the surface of the alginate films due to more complex formation. Therefore films with 1% PLO coating were expected to have a much slower release compared to films coated with 0.5% PLO or 0.1% PLO. However the results showed that coatings of different PLO concentrations have very little effect on the release profiles of the different proteins.

This could be due to the fact that (i) the pore size in the rate controlling PLO membrane was too large, allowing free diffusion of protein or (ii) the PLO coating did not completely coat the surface of the alginate films leaving areas of the alginate uncovered and therefore allowing free diffusion of protein. While both possibilities are equally likely, the latter seems to have been the likely

problem in this particular project. Observations made during the release studies showed that the alginate films detached from the surface of the silicon wafers within the first hour after placing in 1xPBS. The observations are therefore indicative of an incomplete PLO coat that did not go all the way around the alginate film.

As mentioned earlier the protein lysozyme has an isoelectric point of 11. Therefore at a pH of 6.4 (pH of 0.45 μm filtered 4% alginate solution) it is expected to interact strongly with any negative groups that remain after crosslinking alginate. In comparison chymotrypsinogen has a lower isoelectric point of 9.1, similar to BDNF, and is expected to experience weaker interactions with alginate. Therefore the two different proteins are expected to have different release profiles due to their different isoelectric points. However the results from the study showed that proteins, lysozyme and chymotrypsinogen have very similar release profiles.

During the release studies a quantity of alginate particulate matter was noted in the release medium. To check if the particulate matter had any protein entrapped in it, samples obtained at different time points were either (i) centrifuged or (ii) treated with EDTA. Centrifugation was used to separate the dissolved protein from any particulate matter that might have been present in the release medium. Figures 5.2-5.4 show the amounts of dissolved protein found in the release medium at each time point. As seen from the figures almost all

samples (except controls) show 100% release, meaning all of the loaded protein was released from the alginate films and subsequently dissolved in the release medium.

In the past, studies have used EDTA to dissolve alginate gels, subsequently releasing any protein or cell encapsulated in it (Grandolfo et al., 1993). If the total release of the protein increases following EDTA addition it would provide evidence for the presence of protein in the alginate particulate matter.

Figure 5.5 shows the release curves obtained from the EDTA treated samples and the centrifuged samples. As seen from the figure both centrifugation and EDTA treatment produces similar trends in the release profile. Little difference is noticed in the lysozyme release profile between the centrifuged and the EDTA treated samples. This confirms the fact that the majority of the protein lysozyme in the release medium is found in the dissolved form. Very little protein, if any remained in the alginate particulate matter. This is not surprising since any protein released in the alginate particles would diffuse out very rapidly, unless it was electrostatically bound to the alginate.

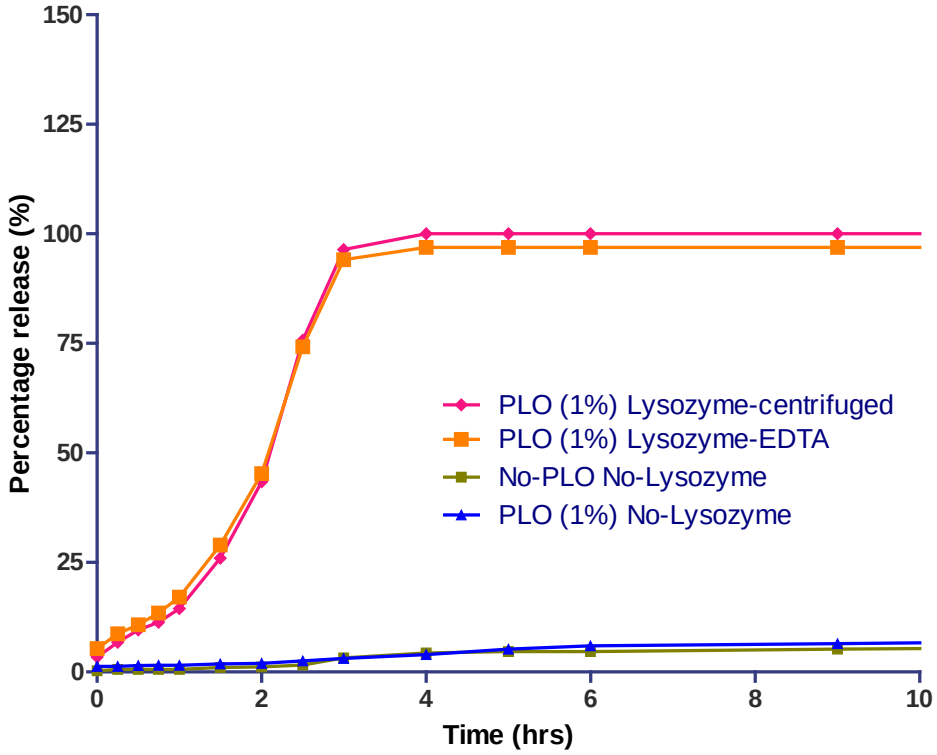


Figure 5.5: Lysozyme and Chymotrypsinogen release from 1% PLO coated alginate films – EDTA treated samples versus centrifuged samples

5.2.4 Poloxamer release profile

The alginate film's ability to encapsulate and release poloxamer-188 (p-188) was evaluated. To track the release of poloxamer FITC labeled p-188 was loaded and released from the alginate coatings.

Figure 5.6 shows the release of poloxamer (p-188) from alginate films over a period of 12 hours. As seen from the figure all films show burst profiles with complete release of p-188 within the first 3 hours. The uncoated film (No-PLO Poloxamer) shows 20% release at time zero, reaching a maximum of 55% at

the 3 hour time point. Similarly all the coated films (PLO (0.1%) Poloxamer, PLO (0.5%) Poloxamer, PLO (1%) Poloxamer) show ~30% release at time zero, reaching a maximum of 70% at the 3 hour time point. Neither the coated nor the uncoated films report 100% release of poloxamer. This could be because of (i) an error in the loading of p-188 or (ii) photobleaching of the FITC attached to the poloxamer. While the first choice is unlikely, photobleaching of FITC seems to be the probable cause.

Alginate debris was again formed during the release study as mentioned earlier. To check if any poloxamer remained in the alginate particulate matter, samples from different time points were either (i) centrifuged or (ii) EDTA treated. Poloxamer release from the two groups however looked similar, implying that all of the released p-188 was found dissolved in the release medium. Very little p-188, if any remained in the alginate particulate matter.

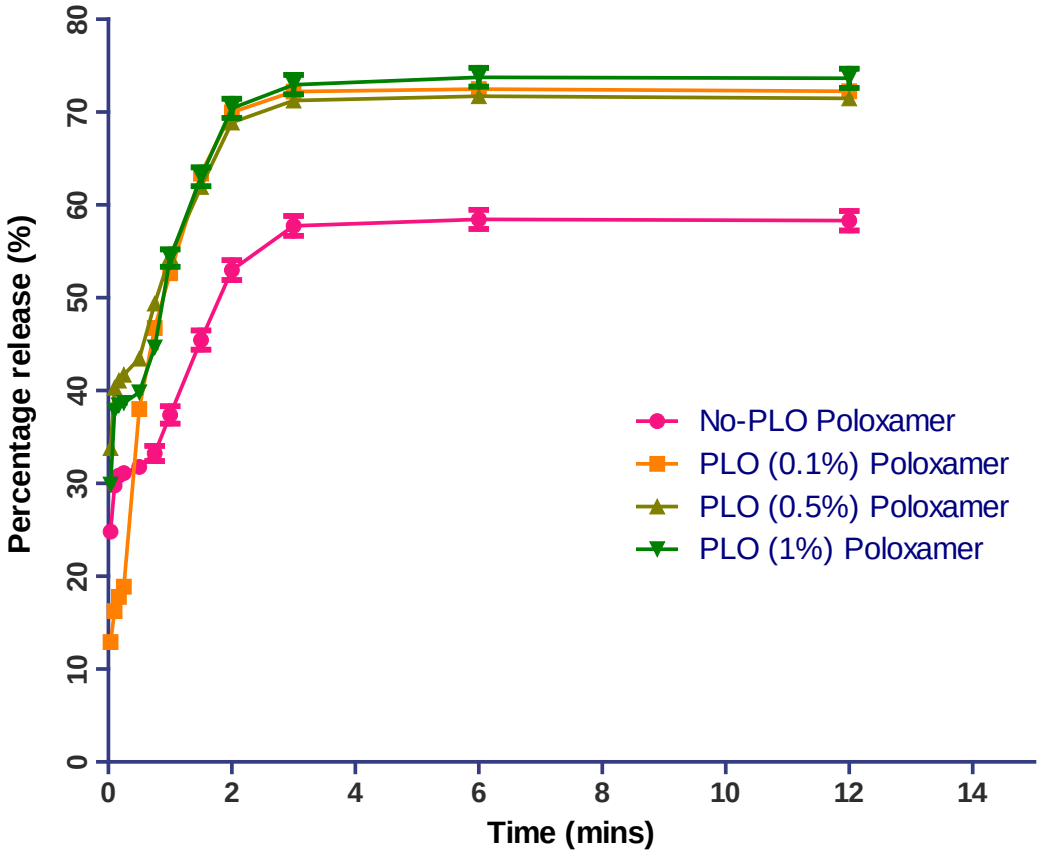


Figure 5.6: Effect of PLO coatings (0.1%, 0.5% and 1% PLO) on the release profile of p-188 in alginate

6 CONCLUSIONS

In conclusion, this present study clearly showed the feasibility of manufacturing alginate coatings for silicon electrodes by a spin coating technique. The silicon can be coated with multiple layers and these final coating can be of a multi valent cationic polymer. The thickness of the coatings can be varied by altering material parameters (concentration, viscosity) and/or spin coating parameters (velocity of rotation and speed of rotation). Results from the study clearly showed that coatings ranging anywhere from 5 μm - 80μm could be produced by placing layers of alginate on top of one another.

Results from the protein-solubility test showed that alginate-protein interactions exist for high pI proteins like BDNF (pI 9.1). However, loading of proteins into the alginate coatings can be accomplished by rehydrating the dried alginate coatings using small volumes (100-200μl) of concentrated protein solution.

The results from the in-vitro release studies showed that physiologically relevant quantities of neurotrophins can be loaded and released from the alginate coatings. Also, the alginate coatings manufactured using spinner coating techniques are versatile. Apart from delivering proteins, the coatings can also be used to delivery other neuroprotective substances like poloxamer-188.

7 RECOMMENDATIONS

- One of the major problems that was encountered during the release studies was the dissociation of the alginate films from the surface of the silicon wafers during protein release. The PLO coated alginate films were found to dissociate from the silicon wafer substrates within the first 30 minutes of release. Therefore protein diffusion continued unabated even in the presence of PLO coatings. An electropositive coating on the surface of the silicon wafer could be applied to prevent this problem. Either polyethyleneimine coatings of PLO coatings on the silicon wafers could be used.
- Only three different concentrations of PLO were tested in this project. A more concentrated solution of PLO, >1% (w/v) could be tested to see if more concentrated PLO can prolong protein release.
- The in-vitro release studies showed that complete release of proteins occurred within the first three hours. It is possible that in-vivo release profiles in the brain may be longer from the in-vitro release profiles performed in 1xPBS at 37°C. Therefore electrodes coated with alginate, loaded with either protein or p-188 should be tested in a tissue phantom (e.g. agarose) and then in-vivo.
- The brain extra cellular matrix consists mainly of proteoglycans. Hyaluronic acid (HA) which is a natural component of the brain could be used as an alternate to alginate. HA hydrogels can be investigated for their ability to provide controlled release of neurotrophins.

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APPENDIX A: CALCULATIONS FOR PROTEIN LOADING

Study details	References				
	(Giehl M. K., 1996b)	(Gillespie N. L., 2003)	(Tuszynski et al., 1996)	(Hammond, 1999)	(Vavrek et al., 2006)
Neuron Type	corticospinal neurons	auditory neurons	hypoglossal nerve	corticospinal neurons	corticospinal neurons
Volume of Injury Induced (mm^3)	52	N/A	N/A	52	N/A
When did delivery start?	Immediately after injury	5 days after ototoxin exposure	Immediately after injury	Immediately after injury	Immediately after injury
Amount of BDNF delivered (µg/hr)	0.5	0.016	0.05	0.5	0.5
Time of delivery (days)	7	28	14	14	14
End Result	promotes survival	promotes survival	promotes survival	promotes long term survival	promotes connections
Amount of BDNF to be delivered (µg/hr)	0.005	N/A	N/A	0.005	N/A
Total amount of BDNF to be delivered (µg)	0.346	N/A	N/A	0.346	N/A

A literature search for all papers that showed survival of neurons post injury using BDNF delivery was performed. A few prospective papers were identified and were used for further review. Information such as the volume of injury induced, amount of BDNF delivered, length of delivery and end result were tabulated.

Using the information obtained from previous studies the total amount of BDNF that would have to be delivered to rescue neurons injured by the silicon electrode was calculated.

Calculations:

Volume of injury induced by silicon electrode implantation = 0.5 mm * 0.5 mm * 2mm = 0.5 mm^3

Amount of BDNF to be delivered (μg/hr) = (Amount of BDNF delivered from study (μg/hr) * Volume of Injury expected due to silicon electrode (mm^3)/ Volume of Injury induced from study (mm^3)

Total amount of BDNF to be delivered (μg) = Amount of BDNF to be delivered (μg/hr) * 24 hours * 3 days

APPENDIX B: STANDARD CURVES FOR RELEASE STUDIES

The following standard curves were made using known concentrations of lysozyme and chymotrypsinogen.

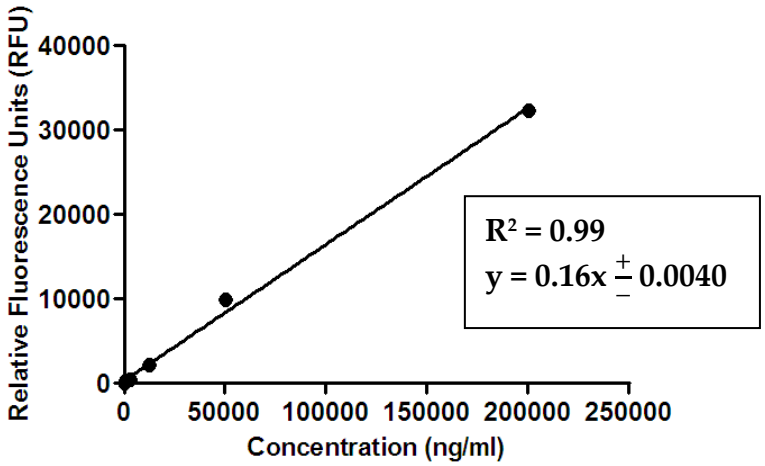

Figure A-1: Standard curve for Lysozyme extending over a range: 48.8 ng/ml - 200 µg/ml

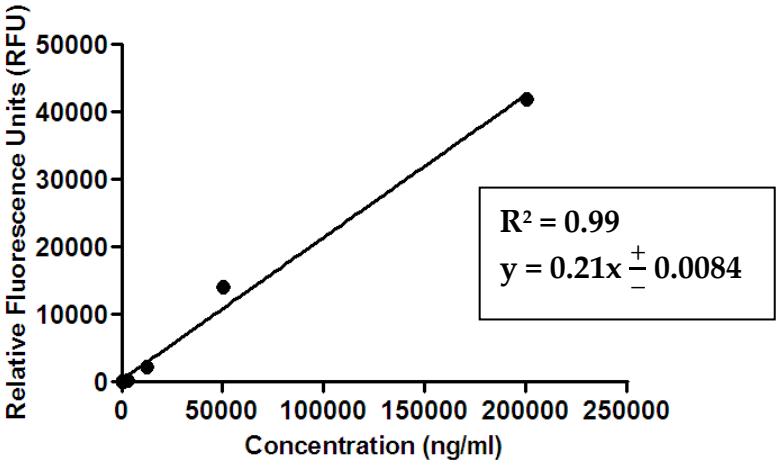

Figure A-2: Standard curve for chymotrypsinogen extending over a range: 48.8 ng/ml - 200 µg/ml

APPENDIX C: PROTOCOL TO MAKE FILTERED 4% ALGINATE

1. Weight out the required amount of alginate using a weighing scale. To prepare 500 ml of 1% alginate solution weight out 5g of pharmaceutical grade alginate power.
2. Take 500 ml of distilled water in a beaker. Preparation of solution can be done using a magnetic stirrer.
3. Since alginate takes a long time to dissolve add the alginate powder in small quantities over intervals of time until all the alginate is completely in solution.
4. Take some of the prepared 1% alginate solution in a 5 or 10 ml syringe. Fit the syringe with a 0.45 μm Nalgene filter. Slowly filter the alginate without applying too much pressure. If sterile alginate is required for experimentation then this should be done into a sterile container and all subsequent steps should be performed in the laminar flow hood using sterile technique.
5. Place the filtered 1% alginate solution in 50 ml tubes and freeze them using liquid nitrogen in a beaker.
6. Once the solution is completely frozen, place the 50 ml tubes in a -80 deg C freezer. Let the tubes sit in the freezer for 3-4 hours.
7. Take the tubes out of the freezer and place them in the freeze drying apparatus. Freeze drying the alginate solution may take anywhere from 3-4 days.
8. Take the free-dried alginate and weight them using a weighing scale. This will help determine if there is any moisture left in the alginate.

9. The 0.45 μm filtered alginate can then be used to prepare a 4% alginate solution. To prepare 50 ml of 4% alginate solution take 2g of 0.45 μm free-dried alginate and dissolve it in 50 ml of distilled water.
10. Use a mechanical stirrer with the blades to prepare the 4% alginate solution. As mentioned earlier add the alginate in small quantities to ensure complete mixing.

APPENDIX D: PROTOL FOR CLEANING SILICON WAFERS

1. Break the wafers carefully. Use a diamond cutter or just use of the end of the spatula.
2. Break a single silicon wafer from the manufacturer into a number of smaller 2 cm x 2cm wafers. The dimensions of the wafers need not be exact. Normalization of all data can be done at a later time to account for the different sized wafers.
3. Place the wafers inside a 1000-1500 ml beaker. Fill it up with 250 ml of distilled water.
4. To the distilled water add 50 ml of Ammonium Hydroxide and 50 ml of Hydrogen Peroxide (30%). The ratio of water, ammonium hydroxide and hydrogen peroxide should be 5:1:1.
5. Make sure that all the wafers in the beaker are completely submerged in the alkali solution. Add a little distilled water if required to keep the wafers submerged completely.
6. Let the beaker sit on a hot plate until it boils.
7. Let the solution boil for 5 mins. Make sure that the temperature of the solution does not go above 72 degrees C.
8. Wash the beaker/wafers at least 4 times with distilled water. Make sure that there is no ammonium hydroxide left in the beaker or the wafers.
9. Now repeat the same process with water, concentrated Hydrochloric acid (conc. HCL), hydrogen peroxide (30%) in the ratio 5:1:1.
10. Allow the solution to boil for 5 mins.

11. Rise with distilled water at least 4 times to completely remove any HCL or peroxide that may be present in the wafers.
12. Let the wafers air dry overnight.
13. Wipe the wafer clean using wipes.

APPENDIX E: SPINNER COATING ALGINATE ON SILICON WAFERS

1. Solutions of 2% and 4% alginate filtered using 0.45 μm Nalgene filters were prepared as mentioned earlier.

   Note: You will need to have pre-cleaned wafers available before starting this procedure. The silicon wafers are cleaned using the RCA cleaning procedure mentioned above.
2. Take a 1 ml syringe and fill it up with the 2% or 4% alginate solution.
3. Open the lid of the spinner coater and place a clean silicon wafer on the disk. Make sure that the silicon wafer is centered. This will be important to ensure that an even coating is formed on the surface of the silicon wafer.
4. Open the air supply connected to the vaccum pump. Once the flow of air has been established turn on the vaccum pump attached to the spinner coating apparatus. The vaccum pump will help hold the silicon wafer in place during the coating procedure.
5. Slowly cover the surface of the silicon wafer using the alginate solution. Care should be taken to avoid any bubbles from forming during this procedure. If bubbles are formed use a plastic Pasteur pipet to remove them from the surface.
6. After application wait for 1-2 minutes for the alginate solution to spread evenly on the surface of the silicon wafer.

7. In the meantime input the required parameters such as (i) speed of rotation (ii) acceleration and (iii) time of spinning into the spinner coating apparatus.
8. Run the spinner coater by hitting the "RUN" button.
9. Wait for the spinner coater to stop completely before opening the lid.
10. Turn off the vaccum pump. Use forceps to gently remove the silicon wafer from the spinning stage.
11. Let the alginate film air dry by placing it outside. Other methods include blow drying the surface using forced air or placing the alginate on a hot plate.
12. Slow drying of the alginate is better since fast drying could produce cracks on the surface of the film.
13. However when multiple layers of alginate need to be spin coated (for example 3 or 4 layers), the 3rd or 4th coat may take a very long time to dry. At these times a hot plate could be used to speed the drying process. However it is important to ensure that the heat is kept low. Cracks will form when if the temperature is too high or if the wafer is kept on the hot plate for a very long time. At any time some amount of moisture should remain in the film to keep it intact. Complete drying of the film will produce cracks making it un-usable for release studies.
14. After completion of multiple/single layer(s) of alginate crosslinking of the alginate is done using a 10% $CaCl_2$ solution (10 g of $CaCl_2$ dissolved in 100 ml of distilled water).

15. The 10% $CaCl_2$ solution is sprayed on the surface of the films using a spray bottle. Use 4-5 sprays using a 3 oz spray bottle or enough to cover the entire surface of the alginate thin film using $CaCl_2$ solution.